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THE ANALYSIS AND BREAKDOWN DURING CANNING OF FLAVOR ENHANCERS

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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Peter Spink

Supervisor

R. A. Lovett

Date Sept. 18, 1984

Dedicated to

- my beloved Parents

ABSTRACT

A high performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of the flavor enhancers monosodium glutamate (MSG), inosine-5'-monophosphate (IMP) and guanosine-5'-monophosphate (GMP) and chloride (NaCl) added to food, as well as other related compounds present in food such as pyroglutamic acid and aspartic acid. This rapid method exhibited excellent recoveries, and did not require derivatization or gradient elution by using refractive index (RI) and ultra-violet (UV) detectors in series.

The stability of flavor enhancers in the canning process was studied under various pH conditions. It was found that MSG was stable while the flavor nucleotides IMP and GMP were hydrolyzed. The corresponding nucleosides and bases; inosine and hypoxanthine, guanosine and guanine were shown to be the decomposition products of IMP and GMP, respectively.

ZUSAMMENFASSUNG

Es wird eine HPLC-Method zur Untersuchung von Nahrungs-mitteln beschrieben, welche die gleichzeitige Bestimmung von Geschmacksverstärkern Glutamat (MSG), Inosin-5'-monophosphat (IMP) und Guanosin-5'-monophosphat (GMP) neben zugesetztem Chlorid (NaCl) und vorhandenen Verbindungen wie Pyroglutaminsäure und Asparaginsäure erlaubt. Diese schnelle Methode ist hervorragend reproduzierbar und erfordert weder eine Derivatisierung noch eine Gradientenelution bei Verwendung einer Kombination aus Refraktometer und UV-Detektor.

Die Stabilität von Geschmacksverstärkern beim Konsevierungs-prozess von Nahrungsmitteln wurde unter verschiedenen pH-Be-dingungen untersucht. Es wurde festgestellt, daβ MSG im Gegensatz zu den geschmackunterstützenden Nukleotiden sehr stabil ist. Es konnte gezeigt werden, daβ die korrespondierenden Nukleoside bzw. Basen Inosin und Hypoxanthin, Guanosin und Guanin Zersetzungs-produkte des IMP bzw. GMP sind.

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1. INTRODUCTION

A flavor enhancer is defined as a substance which when present in a food accentuates the taste of the food without contributing any flavor of its own (73). Monosodium glutamate (MSG) for many years has been the best known and most widely used flavor enhancer. The nucleotides today are commonly used together with MSG because of their synergistic taste effect. There are over 8000 scientific publications on flavor enhancers (105) which show that flavor enhancers, including monosodium glutamate (MSG), inosine 5'-monophosphate (IMP) and guanosine 5'-monophosphate (GMP) are among the most studied food additives.

Although, MSG was listed as a GRAS (generally regarded as safe) substance for many years (106), questions concerning its safety arose from time to time and the use of MSG is still controversial. Since flavor enhancers are additives in many foods and with the public concern over food additives, there is a need to develop analytical methods to quantitate flavor enhancers for product control. Also, using these methods one can investigate the stability of these compounds in prepared foods.

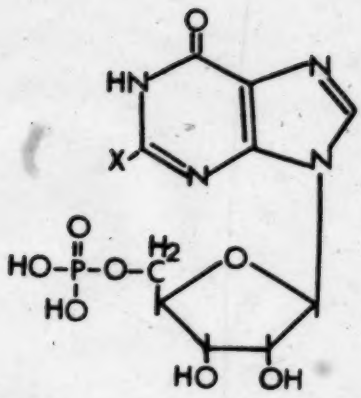
In an attempt to introduce information related to the many aspects of flavor enhancers, the following chapter is divided into six sections. The first five sections deal with 1) general interest in flavor enhancers; 2) toxicological aspects; 3) compounds related to flavor enhancers; 4) stability of flavor enhancers (MSG, IMP and GMP); and 5) analytical methods. The sixth section is devoted to high performance liquid chromatographic (HPLC) methods.

Since flavor enhancers are added to many canned foods and since canning is such a vigorous processing procedure, canning was investigated to determine the stability of flavor enhancers. Canning was carried out at various pHs and times which mimicked usual commercial canning procedures. A detailed review of all the aspects of canning is beyond the scope of this thesis. Interested readers are referred to the reference 117.

1.1 General Interest

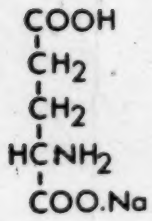
1.1.1 History of Flavor Enhancers

The story of MSG began centuries ago when certain seaweeds were used in the Far-East to improve the flavor of soups and other foods (73). The function of MSG as a flavor enhancer was discovered by Ikeda in 1908 at the University of Tokyo when he was carrying out research into the kelp, seatangle (*Laminaria japonica*) (87). Later, under Ikeda's direction, Kodama in 1913 isolated and identified the histidine salt of inosine monophosphate from dried bonito (101). As an aside, it is interesting to note that MSG and IMP were actually first isolated by two Germans: Ritthausen who isolated MSG in 1886 and Liebig who isolated IMP in 1847 (73). Nevertheless, it was the Japanese scientists who discovered the significance of these flavor enhancers. Kuninaka between 1951 and 1958 pointed out that although inosine monophosphate can exist as 2', 3'and 5'-isomers, only inosine-5'-monophosphate (IMP) possessed flavor activity. He also discovered that guanosine-5'-monophosphate (GMP) possessed flavor activity which is more marked than that of IMP. Later, GMP was reported to be the main flavor enhancing component of dried mushroom "shiitake" (106). Disodium IMP as well as disodium GMP were approved by the Food and Drug Administration (FDA) in 1962 as food additives. They were then used along with MSG as the flavor enhancers, and so IMP and GMP were actually called flavor nucleotides to distinguish them from other nucleotides (103, 106). The term "flavor nucleotides" is used throughout this thesis to indicate IMP and GMP. It should also be noted that only L-glutamic acid possesses flavor enhancing properties, the D-form has no flavor activity (106). The nucleotides, in addition to their effectiveness at much lower concentration (especially when used together with MSG), have been found to be superior to MSG for certain types of foods (addition to high protein foods, for example). It also has been observed that the nucleotides tend to create a sense of increased viscosity providing more body, to soups, for example (73) and masking the undesirable flavors (104). The chemical structures of MSG, IMP and GMP are shown in Figure 1.1.



IMP : X = H

GMP : X = NH_2



MSG

Fig. 1.1 Chemical Structures of The Flavor Enhancers

1.1.2 Uses of MSG

Since professor Ikeda's discovery of MSG, it has been used extensively all over the world as a flavor enhancer (210). MSG is used in soups, broths, sauces, gravies, spice blends, as well as in a wide variety of canned and frozen food stuffs (8). Some researchers believed that MSG can maintain freshness and it may restore natural glutamates lost during processing (77).

In Japan, MSG is also added to sake (Japanese wine) and table salt. Table salt is coated with approximately 10% powered MSG to prevent the caking tendency of the salt and to enhance the savory taste (210).

In the United-States, MSG has been used as a swine feed additive (210). Researchers found that animal appetite increased when MSG was added to feed. MSG is sometimes used with ordinary sugar to improve the palatability of bitter drugs (210).

In the field of medicine, there are several interesting applications: MSG has been used for the successful treatment of coma due to hepatic failure and for certain types of epilepsy (99). A study at the University of California has indicated that there is a glutamine deficency in cases of multiple sclerosis and MSG is being used in the treatement of this disease (99).

1.1.3 Manufacture and Production of Flavor Enhancers

1.1.3.1 MSG

One year after Ikeda's discovery of MSG, it was produced industrially (109). MSG can be produced in three ways: 1) Chemical hydrolysis of substances containing large amounts of glutamate, such as gluten (99); 2) Fermentation by microorganisms which can enzymatically produced glutamic acid from several carbon sources such as acetic acid (99), glucose, etc. (140); 3) Chemical synthesis with a method developed by Ajinomoto which utilizes acrylonitrile (CH_2=CH-CN), hydrogen (H_2), carbon monoxide (CO) and ammonium cyanide (NH_4CN) to produce racemic glutamic acid. Subsequently, L-glutamic is optically resolved and the acid converted to MSG using sodium hydroxide (99).

Among the three methods, fermentation is the main method of production, whereas the hydrolysis method had been exclusively employed for the first 50 years. The chemical synthesis method has been abandoned by Ajinomoto (99), mainly because of consumer concerns with "artificial" MSG.

After World War II, demand for MSG increased rapidly, not only in the United-States, but also in Europe. Thus, production and sales of MSG which was mainly confined to Japan and the Far-East before the war, expanded its market over the world after the second world war (140). Several US companies are now manufacturing MSG. Today 60% of the world production of MSG is manufactured by fermentation, 25% is made from vegetable protein (mainly in Japan), and 15% is derived from beet sugar wastes (in US and Europe) [140]. Japan provides about one third of the total world production (Table 1.1). One fourth of this is exported. In other words, almost 25% of total global production is consumed in Japan.

1.1.3.2 Flavor Nucleotides

The manufacturing of flavor nucleotides is mainly in Japan, dominated by five companies: Yamasa; Takeda; Ajinomoto; Kyowa Hakko and Asahi Chemical. Four independent methods have been used: 1) Enzymatic degradation of RNA; 2) Combination of nucleoside fermentation and chemical phosphorylation; 3) Direct nucleotide fermentation; and 4) Chemical decomposition of RNA into nucleosides and their chemical phosphorylation (106). Details of manufacturing methods have not been described.

The production of IMP and GMP increased tenfold during the first ten years after the discovery of their flavor activity in 1958 (106). After the approval of the US Food and Drug Administration in 1962, the use of IMP and GMP as food additives was assured and they were listed in the GRAS list. The monthly production capacity of flavor nucleotides in Japan is shown in Table 1.2.

TABLE 1.1

Monthly Production Capacity of MSG in 1979

Area	Tons
Japan	9,700
USA	2,000
Taiwan	4,100
Italy	1,200
France	2,500
Spain	200
West Germany	200
Hong Kong	50
Korea	4,500
Thailand	2,050
Malaysia	340
Philippines	900
Indonesia	1,440
Peru	200
Brazil	900
China and others	(1,000)
Total	30,280
	(31,280)

Source from Ref. 106.

TABLE : 1.2

Monthly Production Capacity of IMP and GMP in Japan

Year	Tons	Year	Tons	Year	Tons
1963	37	1968	285	1973	395
1964	42-43	1969	330	1974	427
1965	68	1970	350	1975	463
1966	155	1971	390	1976	463
1967	220	1972	395	1977	463

Source from Ref. 106.

1.1.4 Level of Use of Flavor Enhancers

In many countries MSG alone is marketed to food processing industries as a food additive (210). There are several recommendations for the level of use of MSG in food preparation, because there appears to be considerable variability from person to person as to the optimum MSG level (8). The French recommend using MSG as 1% of salt (NaCl) content added to a recipe (40). In American, results of taste panel studies on processed foods indicated that an MSG level of 0.2-0.8 percent of food (by weight) is optimum (8). For cooks, in homes or restaurants, this translates to one-half or one teaspoonful per pound of meat or per 4 to 6 servings of vegetables, casseroles soups, etc. Japanese suppliers recommend use of 0.2-0.3g of MSG per 100ml of serving (140,210). In food processing, a suggested use of the mixture of MSG and flavor nucleotides is shown in Table 1.3.

1.1.5 Synergistic Taste Effect

Accidentally, Kuninaka in 1955 discovered that there is a strong synergistic taste effect between MSG and IMP (106). Two years later, he found a more marked synergistic effect between MSG and GMP. The synergistic action can be explained in the following examples.

MSG (10g) + IMP (1g) $\simeq$ MSG alone (55g) and,

MSG (10g) + GMP (1g) $\simeq$ MSG alone (209g)

Studies on this synergistic taste have been reviewed extensively (30, 92, 104, 156, 157, 185, 202-206, 208). The effect has also been illustrated diagramatically (Figure 1.2).

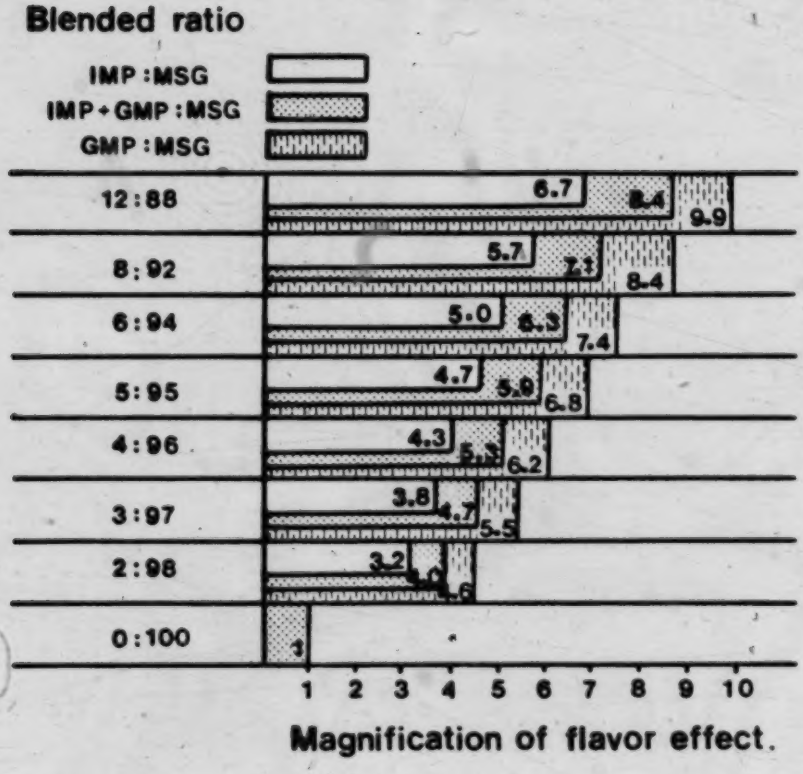
TABLE : 1.3

Suggested Use Levels of Flavor Nucleotides

Processed Foods	Mixtures levels of MSG with IMP, IMP+GMP or GMP required for 10kg			
	MSG (g)	IMP (g)	IMP+GMP(g)	GMP (g)
Dehyrated soups and gravies (Serving dilution:1 to 12-13)	600-800	20-26	12-16	9.0-11
Canned soups and gravies	42-18	0.36-0.54	0.22-0.33	0.15-0.23
Canned foods (poultry, sausages, ham and fish fillets)	10-20	1.0-1.5	0.60-1.0	0.40-0.70
Canned crab	7-10	0.20-0.30	0.10-0.20	0.09-0.13
Canned fish preserved in oil	10-30	0.50-1.0	0.30-0.60	0.25-0.43
Canned asparagus	8-16	0.40-0.50	0.30-0.40	0.10-0.20
Sausages (Frankfurters or Viennas)	30-50	1.2-2.4	0.8-1.4	0.5-1.0
Frozen Foods(hamburgers)	10-15	0.20-0.40	0.10-0.23	0.09-0.20
Ketchup	20-30	3.0-6.0	1.8-3.5	1.3-2.5
Mayonnaise	40-60	2.0-3.0	1.2-1.8	0.9-1.3
Sauces (Worcestershire type)	30-60	4.0-8.0	2.3-4.7	1.7-3.5
Vinegar		10-15	7.0-10	5.0-7.0
Snacks (potato chips,				

peanuts and crackers)	10-50	0.50-1.0	0.30-0.70	0.20-0.40
Soy sauce	30-60	4.0-8.0	3.0-5.0	2.0-4.0
Soup powder for instant noodles(Serving dilution:1 to 60-65)	1,000-1,700	50-90	30-60	13-26

Source from Ref.1



Note:

The results above were obtained by experiments using a 1% salt solution. The flavor effects shown above may change according to the concentration of salt or glutamic acid and/or 5'-nucleotides naturally occurring in foods.

Source from Ref. 6

Fig. 1.2 Synergistic Effect of IMP, IMP + GMP and GMP used with MSG

Kuninaka stated that if the synergistic action between MSG and flavor nucleotides had not been discovered, the latter would not have been used commercially because flavor nucleotides themselves have very weak taste intensity (104, 106). A mixture of MSG with IMP was first sold in Japan as a new chemical seasoning in 1960. At present, the mixtures of MSG with IMP or GMP or both of IMP and GMP are used widely in food processing or in homes (109). The following ratios are generally being used:

$$\text{MSG : IMP} = 88 : 12 \text{ or } 92 : 8 \text{ or } 96 : 4$$

$$\text{MSG : (IMP+GMP)} = 95 : (2.5 + 2.5) \text{ or } 94.5 : (0.5+5.0)$$

The mixture of IMP and GMP with ratio of 50 : 50 has been sold in Japan under a commercial name "Ribotides", or "IG".

1.1.6 Daily Consumption and Recommended Consumption

1.1.6.1 MSG

A large amount of free glutamic acid is present in natural foods (Tables 1.4, 1.5). There is of course, also a considerable amount of glutamic acid ingested in bound form in food proteins (Table 1.8). The total of free glutamic acid consumed daily as MSG [from natural food and added to food (Tables 1.4-1.8)] typically equals about 1/1000 of the total glutamic acid present in the human body, including that in the body's protein (8a). The MSG consumption per capita, and the free glutamic acid in the organs of a normal adult are shown in Table 1.9 and 1.10, respectively.

On the average, about 20 additional grams (2/3 oz) of naturally occurring glutamate is ingested each day as a part of normal food consumption (8a). The ratio of protein bound glutamic acid and free glutamic acid ingested per day varies with food habits depending on protein consumption and MSG used.

TABLE : 1.4

L-Glutamic Acid (mg/100g) in Vegetable Foods

Food Item	L-glutamic acid	Literature
Carrot	3.02	104
Onion	0.69	"
Garlic	1.29	"
Ginger	0.92	"
Egg plant	0.84	"
Pumpkin & Squash	3.03	"
Spinach	3.85	67
Potato	73.8-102	172
Lemon	7.30	104
Apple	3.60	"
Grape	34.3	"
Green tea (new leaf)	208-504	"
Dried tangle	1780-4226	"

TABLE : 1.5

L-Glutamic Acid (mg/100g) in Animal Foods

Food item	L-glutamic acid	Literature
Beef (tenderloin)	33	104
Pork (tenderloin)	11	"
Chicken meat	44	"
Chicken bone	40	"
Duck meat	50	"
Prawn	51	"
Oyster	264	"
Scallop	150	"
Abalone	109	"
Squid	44	"
Fresh sea urchin (66.1% moisture)	280	186

TABLE : 1.6

Flavor Enhancers in Processed Foods

Food item	Total 5'-nucleotides (μM/100g)	MSG (mg/100g)
Canned mushroom, solid	24.7	22
Canned asparagus, solid	17.4	55
Canned green peas, solid	4.9	4
Canned green bean, solid	14.8	18
Canned sweet corn, solid	29.3	100

Source from Ref. 106

TABLE : 1.7

MSG (mg/100g) Content of Processed Foods

Food item	MSG	Analytical methods	Literature
Chicken-in-a-Mug	6850	HPLC/RI	173
Soy sauce	930	"	"
Won-ton soup mix	1860	"	"
Bacon flavored croutons	140	"	"
Soup mixes			
- Mild pepper	10400	GLC/TMS	36
- Cream of mushroom	5000	"	"
- Minestrone	2780	"	"
- Vegetable	10130	"	"
- Chicken noodle	10100	"	"
- Beef noodel	3290	"	"
Bouillon cubes			
- Beef	13100	"	"
- Chicken	10290	"	"
Condensed soups			
- Vegetable	290	"	"
- Vegetable + beef	250	"	"
- Wonton	750	"	"
Restaurant soups			

- Wonton	1170	"	"
- Canellonis (french)	90	Enzymatic	11
- Stew	250	"	"
- Rice with mushroom	500	"	"
Dried foods			
- Instant ham and green peas	2900	"	12
- Chicken vegetables	1400	"	"
- Onion soup	2300	"	"
- Tomato and noodles	2000	"	"
Canned soups			
- Mushroom	16500	"	"
- Asparagus	12500	"	"
- Potato and long onion	12500	"	"
- Fish	5000	"	"
- Chicken noodles	5500	"	"
Pre-Cooked foods			
- Chicken with tomato	376.4	Colorimetric	31
- Rabbit with sauce	179.9	"	"
- Pork tenderloin	132.0	"	"
- Bonito	241.3	"	"
- Hamburger	204.3	"	"
- Meat - ball	287.4	"	"
- Pigeon	143.3	"	"

- Venison	198.3	"	"
- Sausage	135.6	"	"
Baby foods			
- Chicken with vegetables	260	"	59
- Veal and cereals	100	"	"
- Beef and Vegetables	220	"	"
- Corn	310	"	"
- Sheep	160	"	"
Dried foods			
- Instant chicken -flavored broth	12240	Dowex column/ Volumetric	54
- Beef bouillon (granular)	9750	"	"
- Chicken bouillon (cubes)	11340	"	"
- Beef bouillon cubes	10840	"	"
- Vegetable soup mix	7180	"	"
Liquid foods			
- Condensed pea soup	310	"	"
- Condensed chicken broth	1300	"	"
- Condensed chicken broth + rice	320	"	"
- Condensed beef broth	790	"	"
- Canned green beans			

(seasoned)	170	"	"
Shrimp egg rolls	587	Dowex Column/ fluorometric	39
Chicken chow mein	759	"	"
Chicken rice soup (liquid)	60.3	"	"
Frankfurter	423	"	"
Bologna	302	"	"
Chicken bouillon soup (cubes)	4350	"	"
Chicken noodle soup (dry mix)	8350	"	"
New England chowder (dry mix)	4390	"	"
Oxtail soup	2990	HPLC/ fluorometric	198
Chicken soup	5400	"	"
Beef and tomato soup	4990	"	"
Golden vegetable soup	3790	"	"
Diet mushroom soup	9870	"	"
Oxo	5630	"	"

TABLE : 1.8

Glutamic Acid (g/100g) in Food Protein

Protein	Glutamic acid
Albumin (egg white)	16.5
α - Casein (milk)	22.5
β - Lactoglobulin (milk)	20.0
Gliadin (wheat)	45.7
Zein (maize)	26.9
Hordenin (barley)	38.4
Globulin (coconut)	21.8
Arachin (peanut)	20.8
Glycimin (soybean)	20.5
Glutenin (wheat)	24.7

Source from Ref. 67

TABLE : 1.9

MSG Consumption in Selected Countries

Country	Total tons/yr	g/day/capita
Taiwan	18,000	3.0
Korea	30,000	2.3
Japan	65,000	1.6
Italy	8,000	0.4
U.S	28,000	0.35

Source from Ref. 67.

TABLE : 1.10

Free Glutamic Acid in the Organs of a Normal Adult

Tissue	Free glutamic acid (mg)
Muscles	6,000
Brain	2,250
Kidneys	680
Liver	670
Blood Plasma	40
Total	9,640

Source from Ref. 67.

At the joint FAO/WHO Expert Committee on Food Additives Meeting in 1970, the acceptable daily intake (ADI) for MSG was established at 0-120 mg of glutamic acid equivalent per kilogram body weight for humans over the age of 12 weeks (210). In 1973, the committee reaffirmed the ADI for other glutamic acid salts including MSG. Following these recommendations, the Codex Alimentarius Commission of FAO/WHO of the United Nations classified monosodium glutamate and the ammonium, calcium and potassium salts of glutamic acid, in A-1 list of food additives. These additives have been toxicologically cleared for use in food, with the exception of use in baby food for infants under 12 weeks of age (210).

In Canada, MSG is considered to be a food ingredient (7,8) which has functionality as a seasoning and flavoring agent. Hence, it may be used at levels consistent with "Good Manufacturing Practices", provided its presence is declared on the label of prepackaged foods. MSG is also specifically authorized in the standards of identity of many foods under Title 21 of the United States Code of Federal Regulations (CFR) but its presence must still be disclosed on the label (8, 40).

In the late 1960s, the consuming public voiced concern over the possible effects of glutamates fed to infants and small children (see section 1.2.1). At the time, MSG was being added to baby foods to enhance flavor. Because of the concern expressed by the public, baby food manufacturers in both US and Canada voluntarily stopped using MSG in late 1969 (8a). In France, MSG was banned for use in baby foods in 1966 (40).

1.1.6.2 Flavor Nucleotides

5'-Nucleotides have been found widely in common foods, especially in animal foods (Tables 1.11 and 1.12), and in processed foods (Table 1.6). The contents of purines (heterocycles including adenine, hypoxanthine and guanine) including purine bases, nucleosides and nucleotides in some common foods and beverages are shown in Table 1.13. The daily intake of purine compounds from diets could be estimated from these data. It was reported that the amount of purine intake as IMP equivalent was calculated to be 1875mg/day for a Japanese and 2503 mg/day for an American (103). The average daily consumption of flavor nucleotides

TABLE : 1.11.

5'-Nucleotides (mg/100g) in Vegetable Foods

Food item	IMP	GMP	AMP	CMP	UMP
Asparagus, green	-	Trace	3.8	1.9	1.9
Lettuce	Trace	Trace	0.9	-	0.5
Tomato	-	-	10.4	0.5	2.2
Cucumber	-	-	0.5	Trace	0.6
Japanese radish	Trace	-	1.3	"	1.4
Onion	Trace	-	0.8	"	0.5
Bamboo shoot	-	-	1.1	"	1.3
Fresh mushroom, "Shitake"	-	18.5-45.4	21.5-30.2	0.5	13.3-25.3
Common mushroom	-	Trace	11.3	9.0-14.5	6.4
Dried mushroom, "shitake"	-	216	321	-	-
Potato	-	2.1	3.0	-	2.1

Source from Refs. 4, 28 and 106.

IMP = inosine-5'-monophosphate; GMP = guanosine-5'-monophosphate;

AMP = adenosine-5'-monophosphate; CMP = cytidine-5'-monophosphate;

UMP = uridine-5'-monophosphate.

TABLE : 1.12

5'-Nucleotides (mg/100g) in Animal Foods

Food item	IMP	GMP	AMP
Beef	163	-	7.5
Pork	186	3.7	8.6
Chicken	115	2.2	13.1
Whale	326	5.3	2.4
Horse mackerel	323	0	7.2
Sweet fish	287	0	8.1
Common sea bass	188	0	9.5
Pilchard	287	0	0
Black sea bream	421	0	12.4
Pike mackerel	227	0	7.6
Mackerel	286	0	6.4
Keta salmon	235	0	7.8
Tuna	286	0	5.9
Globe fish	287	0	6.3
Eel	165	0	20.1
Dried bonito	630-1310	0	Trace
Squid	0	0	184
Spiny lobster	0	0	82
Squilla	26	0	37

Source from Refs. 4, 103.

TABLE : 1.13

Contents of Purine Compounds in Some Common Foods and Beverages

Food and Beverages	mg Nitrogen in purines/100g fresh sample
Wheat flour	10.2
Rice	11.6
Potato	4.3
Soybean	65.6
Tomato	4
Carrot	3
Cabbage	7
Onion	4
Beef	77.2
Pork	46
Chicken	61
Egg	5
Codfish	49.0
Sardine	89.0
Salmon	76.0
Oyster	57.6
Lobster	88
Coffee	13

Source from Ref. 103

in Japan is estimated to be about 70 mg per capita. According to FAO/WHO (90), the establishment of an acceptable daily intake of flavor nucleotides is not really necessary, because the intake of nucleotides from flavor enhancers is far lower than that from total nucleotides present in common foods and beverages.

1.2 TOXICOLOGICAL ASPECTS

1.2.1 MSG

Schaumburg et al reported an undesirable physiological effect (Chinese Restaurant Syndrome)[1] when excessive amounts of MSG were introduced into the empty stomaches of susceptible individuals (158). Numerous studies using both animals and humans were performed in response to the paper (56, 84, 102, 115, 125, 135, 143-145, 151, 153, 161, 181, 184, 201). Krueger divided the concerns and areas of controversy with MSG's safety as a food additive as follows (105).

1. The production of hypothalamic brain lesions in laboratory rodents following administration of MSG.

2. The production of other physiological changes in laboratory mice, gerbils, guinea pigs and other animals including humans.

3. The production of hypothalamic lesions in primates following administration of MSG.

4. The existence of a "blood-brain barrier" mechanism that acts to protect the body from excess glutamate transfer to the brain where it might cause damage.

5. The existence and prevalence of an idiosyncratic reaction to MSG by some people, commonly referred to as "Chinese Restaurant Syndrome". [1]

---

[1] Chinese Restaurant Syndrome (CRS) is described as "burning", "tightness", and/or "numbness" in the upper chest, neck, and face; beginning shortly after the start of a meal in a Chinese restaurant and lasting less than 4 hours. The symptoms include dizziness, headache, chest pain, palpitation, weakness, nausea, and vomiting (55).

6. The ultimate question of the safety of MSG as a food additive at present and future use levels.

An excellent review on the biochemistry and physiology of glutamic acid was published in 1979 based on the international symposium held in Milan 1978 (55). This review has responded to many of the above concerns with glutamate. Also, many other serious studies on glutamate plasma levels with different glutamate intake from prepared foods have been carried out (175-180).

In the past, studies were conducted to determine if large daily doses of MSG would improve children's intelligence quotients (210). Although results of the studies were not conclusive, no physiological abnormalities were detected even among children to whom 30g of MSG was administered daily. Long term tests with mice and rats showed no differences between growth curves, hematological findings, tissue weights or pathological examinations of test and control animals. Also, it was indicated that no carcinogenic, teratogenic or other reproductive hazards resulted in tests using rats, mice, rabbits and guinea pigs (210). The many years of use of MSG by literally thousands of food processors and many millions of chefs and households without serious deleterious effects also tends to counter consumer concerns (105).

1.2.2 Flavor Nucleotides

The safety of IMP and GMP has been studied using various animal species as follows :

General toxicity : Acute toxicity, subacute toxicity studies and 2-year rat and dog studies were described. The parameters in the prolonged toxicity studies were general behavior, food intake, body weight gain, hematology, blood biochemistry, urine analysis, terminal pathology and histopathology of important organs. All parameters were within normal ranges under the experimental conditions used, except the slight increase of the blood allantoin level in the high dose group which was reported to be due to the normal metabolism of purines (103).

Specific toxicity: Rat multi-generation studies (147) and teratogenicity studies in mice, rats, rabbits and monkeys were described. No significant teratogenicity and no influences upon

the reproductive performance were found (103).

1.3 Compounds Related to Flavor Enhancers

1.3.1 Pyroglutamic Acid

Pyroglutamic acid was first described by Haitinger in 1882 when he heated glutamic acid at 180-190°C (146). One molecule of water was lost giving a new compound called pyroglutamic acid or 2-pyrrolidone-5-carboxylic acid (PCA). The theory and kinetics of this reaction was reviewed in an excellent paper by Wilson and Cannon (199). Further study was reported in the paper by Cleaves (34).

Pyroglutamic acid was found in many processed and stored fruits and vegetables (35, 119-121), in soysauce (91, 110, 142, 209), and was described as having an off-flavor [bitter, medicinal, phenolic, metallic and even burnt] (119, 163, 164). Luh et al found a decrease in pH of thermally processed strained carrots along with the formation of pyroglutamic acid (118). The chemical structure of pyroglutamic acid is as follows:

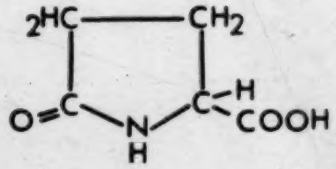


Fig. 1.3 Chemical Structure of Pyroglutamic Acid

1.3.2 Aspartic Acid

One of the two dicarboxylic amino acids present in food is aspartic acid which has similar chemical structure to glutamic acid with one less carbon.

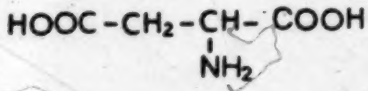


Fig. 1.4 Chemical Structure of Aspartic Acid

Recent biochemical studies suggest that L-glutamate and L-aspartate could be the neurotransmitters of parallel fibers and climbing fibers respectively, in the mammalian cerebellum (43). Since aspartame, a dipeptide (L-aspartyl-L-phenylalanyl-methyl-ester) has a sweetening power of 180-200 times that of sucrose, it has been used extensively in the food and beverage industries (9). It has been suggested that aspartame addition to meals already containing large amount of MSG would result in an early rapid rise in plasma glutamate and/or aspartate concentrations, and increase the potential for dicarboxylic amino acid-induced toxicity (178). Together with glutamate, aspartate also has the potential to show neurotoxicity in large amounts (177).

In some natural foods, the quantity of aspartic acid was found to be as high as glutamic acid (Table 1.14). It would be an advantage to be able to analyse simultaneously for both of these important dicarboxylic amino acids present in food.

1.3.3 Salt (Sodium Chloride)

Salt (NaCl) is the classical and universal flavor enhancer (72). Historically, salt has been used to flavor and preserve meat and fish. In food flavoring, it is second only to sugar in the amount used (72). Americans consume an average of 10-12g of salt per day, of which about 3g occurs naturally in food, 3g are added in cooking and at the table, and 4-6g are added

TABLE : 1.14

Free Glutamic and Aspartic Acid (mg/100g) in Natural Food

Food item	Glutamic acid	Aspartic acid	Literature
Human milk (2nd day)	12.88	2.92	67
Cow's milk (2 month)	0.64	0.08	"
Tomato	140.0	35	"
Potato	73.8	46.8	172
Broccoli	176.0	40.0	67
Mushroom (Psalliota Campestris)	180.0	30.0	"
Grapefruit (white meat)	11.5	87.1	"
Prunes (California)	14.4	185.5	"
Grape (red Malaga)	184.0	12.0	"
Strawberry	44.4	60.1	"
Gruyere de Comte cheese	1050.0	60.0	"

during commercial processing of food (33). Salt improves the flavor of many foods and contributes to palatability and acceptance. Without salt, bread tends to be bland, cheeses are bitter and tart, and processed meats lack texture and flavor (72). In the body, sodium helps maintain proper blood volume and controls the flow of water in and out of body cells. It is also vital for the transmission of nerve impules and the metabolism of proteins and carbohydrates. Chloride is needed to maintain the body's acid balance and for the action of certain enzymes (33).

It was reported that sodium and chloride are not normally retained in the body even when there is a high intake. Amounts consumed in excess of need are excreted, so that the level in the body is maintained within very narrow limits, regardless of intake (73a). Hypertension (high blood pressure) afflicts more than 20% of the world population, with an estimated 24 million cases in the United States in 1980 (73a).

Most of the concern with sodium is centered mainly on possible relationship between salt and hypertension (33, 61, 137, 165). Although medical researchers have not established exactly what causes hypertension (82), there has also been a trend suggesting a replacement of NaCl by another salt, such as potassium chloride, reported Nolan in 1983 (137). In the United-States, at the present time, a number of food companies monitored by US Food and Drug Administration (FDA) have volunteered sodium labeling of their products (61) to implement the "Partial Quality Control Program" suggested by FDA last year. As yet, this program is considered burdensome (61), and the FDA has been looking for ways to streamline it. It seems that an accurate and rapid method for determination of sodium chloride content in processed food along with other flavor enhancers would be useful.

1.4 Stability of Flavor Enhancers

1.4.1 MSG

Hanson et al reported that MSG was very stable under canning conditions after a careful examination of changes of MSG in canned foods with added MSG heated at 240°F(116°C) and a variety of heating times from 20 to 50 minutes. He concluded that no loss in glutamate content was found in canned foods, and glutamate was stable to heat processing(77). Armand et al in a similar experiment carried out on canned mushrooms, asparagus, chicken noodles and various type of sauces, reported 100% recovery of MSG in all samples (11). In spite of these results, the Maillard reaction would be expected to occur between the amino group of MSG and reducing sugars present in some foods. Also, a dehydration reaction in food processing could result in pyroglutamic acid formation. Although the rate of the reaction would be slow, the normal pH of food (pH 3-7) would favor pyroglutamic acid formation (199). These possibilities are summarized in the following equations

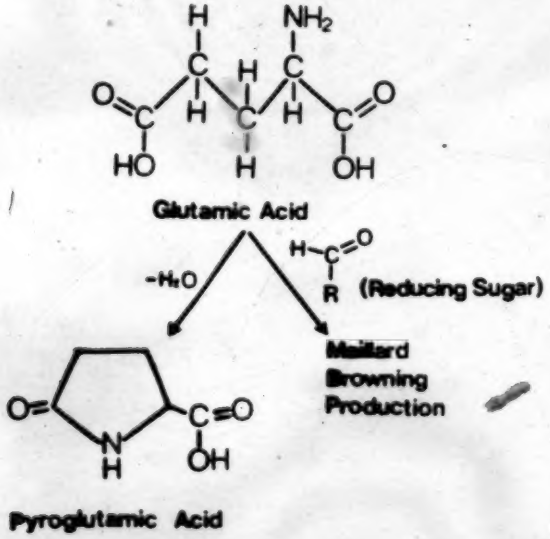


Fig. 1.5 Possible Decomposition of Glutamic Acid

1.4.2 Flavor Nucleotides

Since 1962, flavor nucleotides (IMP and GMP) have been approved as food additives and used widely in food processing, reviews on the stability of these compounds (3, 5, 79, 80, 103, 107) and their application in food (111) have been reported. It was stated that 5'-nucleotides were stable against heat under dry conditions, even mixed with acidic or alkali substances (3). In canning of corned beef, the recovery of a mixture (50:50) of IMP and GMP was reported to be 98 and 94% after 30 minutes heating at 110 and 120°C, respectively (3). The experiments on stability of flavor nucleotides was carried out at various pH conditions, heating times and temperatures. The recovery at pH 5, heating 100°C for 2 hours was reported to be more than 90 and 80% for IMP and GMP, respectively (5). Hashida et al found the remaining flavor nucleotides added to canned seafoods was from 52 to 61% (without reporting the canning conditions). The low percent recovery obtained in canned liquid was accompanied by an increase in nucleotides in the canned solid material and it was assumed that the 5'-nucleotides added were slowly penetrating the solid matrix of canned foods (80). Also, in other experiments with canned vegetables, Hashida et al. found the recovery of 5'-nucleotides in canned mushrooms was from 73 to 74% after 58 days storage. They summarized by saying the 5'-nucleotides were retained fairly well in canned products even stored for 6 months at room temperature (79).

1.5 Analytical Methods

1.5.1 Methods for MSG Analysis

There have been many methods developed for the determination of MSG in foods (11-13, 31, 36, 39, 54, 59, 94, 173, 186, 189, 198). Some steps are similar, such as extraction of the sample with water, purification using clarifying agents, and/or column chromatography for fractionation.

1.5.1.1 Volumetric method

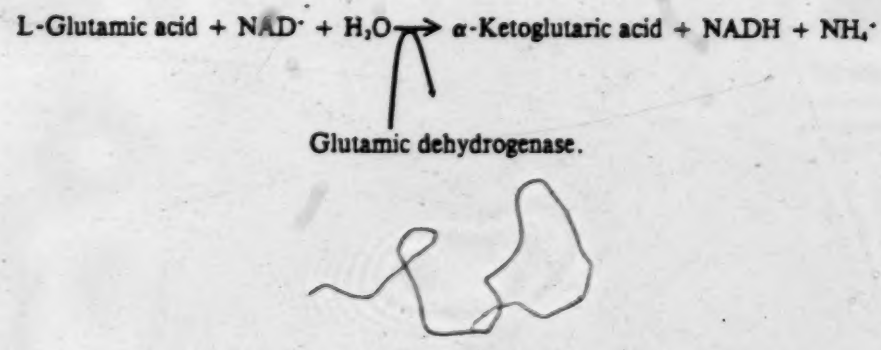
The Association of Official Analytical Chemists (AOAC) method for MSG (139) was originally described by Fernandez Flores et al (54). This procedure for quantifying MSG utilized an extraction of food samples with water and/or with a mixture of acetone/water if a significant amount of starch was present, clarification using charcoal, filtration, evaporation and dilution to volume. The prepared solution was introduced to an anion exchange column, glutamic acid was retained on the column and eluted later with an HCl solution. The amount of glutamic acid in the eluate was then determined using Sorensen formaldehyde titration procedure. MSG was calculated using a conversion factor of 1.15 from amount of glutamic acid obtained.

1.5.1.2 Paper Chromatographic Method

Bailey and Swift proposed a method for estimation of MSG using ascending paper chromatography (13). The sample was extracted with water, filtered and spotted on a paper chromatogram. The paper sheet was developed overnight by ascending chromatography in a solvent mixture of n-butanol-acetic acid-water. Ninhydrin stain was used as the color developing agent. The purple spots were compared with standards of known concentration.

1.5.1.3 Enzymatic method

The enzymatic analysis (11, 12, 94) was based on the Bernt-Bergmeyer method (21), which is an enzymatically catalyzed procedure dependent on the production of NADH as follows:



To displace the equilibrium to the right side of the reaction, the ketoglutaric acid is fixed with hydrazine and NAD^+ was used in excess, in an alkaline medium. The product was measured by observing the increase in optical density at 340 or 365 nm due to the NADH formation.

Another enzymatic method has been reported recently by Kusakabe et al where glutamate in soysauce was determined using L-glutamate oxidase resulting in α-Ketoglutarate, hydrogen peroxide(H_2O_2), and ammonia (NH_3). Spectrophotometric measurement was used for determination of α-Ketoglutarate or hydrogen peroxide derived (112).

1.5.1.4 Fluorometric Method

Coppola et al reported a fluorometric method (104) which involved separation of glutamic acid using column chromatography, and subsequent fluorometric determination of glutamic acid. The derivatization was carried out using 4-phenylspiro-[furan-2(3H), 1'-phthalan)-3,3'-dione] (188), commonly known as fluorescamine. Fluorescamine reacts with glutamic acid forming a fluorophor. The intense fluorophor had to be measured within 30 minutes using a fluorescence spectrophotometer at 366 nm excitation and 480 nm fluorescence.

1.5.1.5 Colorimetric Method

Font and Lopez extracted MSG from baby foods (59) according to the AOAC official method (136). The extracted solution reacted with ninhydrin to develop a colored product. The purple colored product was measured at 560nm. Carballido et al determined the amount of MSG in several pre-cooked foods (31) using yet another colorimetric method based on the reaction of glutamic acid with nitrite, hydroxylamine and $FeCl_3$ as summarized in the Figure 1.3. The colored product was then measured colorimetrically at 490 nm.

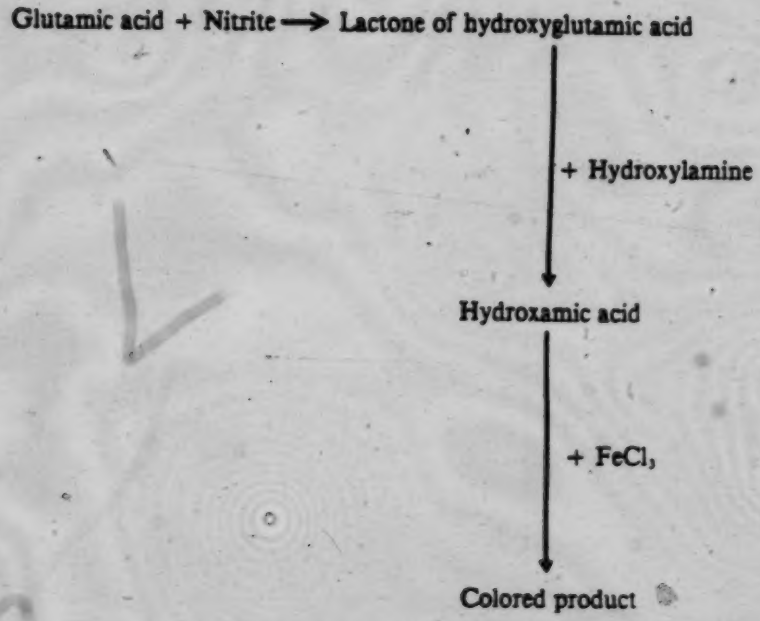


Fig. 1.6 Colorimetric method of determination of MSG

1.5.1.6 Gas Liquid Chromatographic (GLC) Method

MSG contained in processed foods (36) and sea food (186) was extracted with water and purified using an ion-exchange column. Derivatization was carried out with N-trifluoroacetic-n-butylester and/or trimethylsilyl-diethylamine. The derivatized glutamic acid was measured using GLC.

1.5.1.7 HPLC Method

Williams, Winfield, Van de Haar and Cornet described a gradient solvent system in conjunction with a reversed-phase column and a fluorescence detection for analysis of MSG in processed food (198) and mixtures of flavor enhancers (189). MSG was derivatized with dansyl chloride 5-dimethyl aminonaphthalene-1-sulphonyl chloride in a precolumn. The dansylated glutamic acid was detected with an excitation wavelength of 328 nm and an emission wavelength of 530nm. Finally, the HPLC method using an isocratic solvent with a strong anion exchange

(SAX) column and a refractive index (RI) detector was described by Sporns (173). This method did not require the derivatization of MSG, extracts from food samples were filtered and injected directly into the HPLC system.

1.5.2 Methods for Nucleotide Analysis

1.5.2.1 Extraction

Analysis of 5'-nucleotides has been reported by a number of authors. Generally, the extraction step is similar. A homogenized sample was added to cold perchloric acid (21, 79, 80, 98, 196, 207) or to boiling water (116, 131-133, 189) and 5'-nucleotides were extracted into the liquid phase, centrifuged and the supernatant collected. Determination of 5'-nucleotides contained in the filtrate was then carried out using various methods.

1.5.2.2 Spectrophotometric Method

A column chromatographic method consisted of a stationary phase of a Dowex 1 (Cl^-, anion exchange resin) column and a mobile phase mixture of formic acid and sodium formate which separated the individual 5'-nucleotides. The effluents were identified and quantified according to the absorption spectra of individual 5'-nucleotides (131, 207) and their extinction coefficients. A correction factor determined as percent recovery of standard compound was used for the final calculation (207). However, it has been reported that (19, 93) in the separation using an anion exchange resin, 5'-nucleotides could not be separated from 2'- or 3'-isomers. Therefore, these methods were not specific for analyses where 2'-, 3'-nucleotides might interfere (116).

1.5.2.3 Enzymatic Method

5'-Nucleotidase from bull semen was used to liberate the inorganic phosphate (79, 80, 131-133, 169). The amount of liberated phosphate was determined according to the Fiske - Subbarow method (58). The parent 5'-nucleotides were then converted by means of the amount of inorganic phosphate liberated. This method was applied for determination of total

5'-nucleotides present in foods (79, 80, 131-133).

1.5.2.4 Colorimetric Method

Lento et al proposed a colorimetric method for determination of 5'-nucleotides (116). This method involved: 1) a gradient elution system of formic acid/sodium formate on a Dowex 1 anion exchange resin column; 2) a UV spectrophotometric determination of individual 5'-nucleotides eluted, and 3) an oxidation reaction of nucleotides witth periodate. Finally, 2,4-dinitrophenylhydrazine (2,4-DNPH) was used to react with the oxidation products to give yellowish colored compounds. The colored products were measured colorimetrically at 435 nm. The 2' and 3'-isomers did not interfere (116) with 5'-nucleotides in the analysis procedure (not oxidized).

Japanese workers used another method of determination of nucleic acid components (50, 159). Since guanine reacts with Folin phenol reagent giving a blue color, this reaction has been applied to the quantitative determination of guanosine in combination with nucleoside phosphorylase (136). Hypoxanthine reacts with N-1-naphthyl ethylenediamine hydrochloride [Bratton - Marshall (BM) reagent (22)] after reduction with zinc dust and diazotization with $NaNO_2$ to give a red color. The official Japanese method (88, 89) for determination of IMP present in commercial products containing a mixture of flavor enhancers is based on these principles. IMP is decomposed to hypoxanthine by acid hydrolysis after a long period of heating. Liberated hypoxanthine is color developed according to the procedure mentioned above. The reddish solution is measured colorimetrically at 515 nm. Kusuwi et al proposed a modified method (113), where IMP was decomposed to a diazotizable amine (DA) by treatment with zinc powder in HCl. The DA formed a reddish-purple colored derivative with BM reagent and was measured at 540 nm. All these colorimetric methods for the identification of IMP have a large amount of interfering compounds which can be present in food (113). These include metals, amino acids, sugars, ascorbic acid and other nucleotides. Also, these methods cannot distinguish IMP and its corresponding nucleoside and base (113). Thus, they can not easily be applied for determination of IMP present in foods.

1.5.2.5 HPLC Method

Recently, HPLC determination of 5'-nucleotides in food has been reported by a number of workers. Currie et al separated IMP from other nucleic acid substances present in beef using a Whatman SAX column and a gradient elution system with varying pH buffers in combination with UV detection (44). Van de Haar and Cornet analysed IMP and GMP (in a mixture of flavor enhancers containing MSG, IMP and GMP) using a Lichrosorb 10 RP8 column and a gradient elution system of acetonitrile/acetyltrimethyl-ammonium bromide, [MSG was analysed in another procedure] (189). In evaluation of IMP and hypoxanthine as indicators of bacterial growth in stored meat, Parris et al (1983) used a reversed-phase chromatography column (μ-Bondapak C_{18}) with an UV detector (149).

1.5.2.6 Other Methods

An immobilized enzyme method for determination of IMP in fish has been reported by Watanabe et al (196). The enzyme sensor for IMP consisted of three immobilized enzymes (nucleotidase, nucleoside phosphorylase, and xanthine oxidase) and an oxygen electrode. The enzymes were immobilized on a membrane prepared from cellulose triacetate, 1,8-diamino-4-aminomethyloctane and glutaraldehyde. The amount of IMP was determined from the difference between output currents corresponding to the total amounts of hypoxanthine and inosine determined by the inosine sensor (194, 195) and those of hypoxanthine, inosine and IMP measured with IMP sensor.

1.6 HPLC

HPLC, described as high performance (and/or) high pressure liquid chromatography, was developed in the 1960s (53, 114, 152). This technique can achieve high resolution separations with the use of uniform microparticulate chromatographic supports and well designed equipment. There are several excellent reviews on HPLC systems and analysis methods (14, 29, 46, 52, 66, 69, 74, 76, 78, 86, 100, 122, 128, 129, 160, 162, 171, 182, 190, 197, 211). The following sections will focus on HPLC columns and the detection systems used for this

thesis.

1.6.1 Columns

The column is considered the "heart" of the analytical separation system in HPLC. The efficiency of chromatographic separations is improved by using packing materials of uniform size. Small particles, 3-10 micron diameter, are used for porous materials such as silica gel or alumina, while larger uniform particles, 30-60 micron diameter, made of glass coated with silica gel or alumina can also be used, the larger particles require larger columns due to the smaller amount of adsorbant material per unit volume. These types of column packing materials, however, introduce several important constraints on the chromatographic system. Since liquids are viscous and exhibit lower diffusion rates than gases, for example, the columns must be operated at high pressures. HPLC columns are usually made of stainless steel, 25 to 50 cm in length and 2 to 6 mm internal diameters, (for small porous materials)with linear flow velocities of the mobile phase of about 20cm/min (76). An HPLC column is closed and reusable, and hundreds of analyses can be run through a single column without repacking. All the main types of liquid chromatography are now available in HPLC, where columns are packed with silica gel, alumina or organic polymers : partition chromatography or liquid-liquid chromatography; liquid-solid chromatography; exclusion chromatography or gel permeation chromatography; and ion-exchange chromatography.

Ion-exchange chromatography has been used for the analyses in this study. Ion-exchange has a long history of use in analysis (51, 114, 152) including the analysis of amino acids (75), nucleic acid components (39) and nucleotides (18, 23, 32, 57, 78, 81, 96, 130, 170). A substantial improvement in ion-exchange chromatography was made in 1960 by Hamilton (75), who was the first to recognise clearly the benefit of using small particles and high pressure (114). An advance on the original homogeneous resin beads was made by Horvath, Preiss and Lipsky in 1967 (85) with the introduction of pellicular ion-exchange materials, in which glass beads were coated with a thin layer of ion-exchange resin. Kirkland

Zipax ion exchangers (97), in which a polymer was deposited in a porous layer on a glass bead. The mass transfer properties of these materials were reported to be better than those of homogeneous resin beads of the same size, and the materials were incompressible (100). The pellicular materials are now, however, mainly of historical interest (100), and the highest efficiencies (with more active material per unit volume) are obtained with homogenous bonded materials in which the ion-exchange groups are chemically bonded to a small spherical organic polymer or by coating uniform small particles of porous silica gel (42).

Two types of ion-exchange columns, strong anion and strong cation exchangers were employed in this research. The most common type of strong anion exchange (SAX) resin contains quaternary ammonium salts (-NR^+,X^-) obtained by alkylation with trimethylamine (62) as follows :

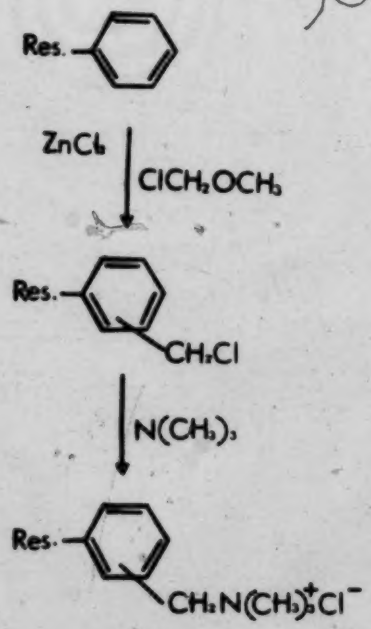


Fig. 1.7 Strong Anion Exchange Resin

The quaternary ammonium material would be considered a strong anion exchanger (to differentiate it from weaker bases such as amine group ($-NH_2$) attached to a solid support). The anion (X^-) associated with the quaternary ammonium salt can exchange with another anion in the mobile phase. The rate of exchange depends on the concentration of the anion in the mobile phase and the relative affinity of the anions for the quaternary ammonium group (38, 62, 66, 86, 123, 192, 193, 200, 211).

Cation exchange packings are based on acidic functional groups such as the sulfonic acid group ($-SO_3^-Y^+$) attached to a solid support. The sulfonic acid material would be considered a strong cation exchanger (SCX) to differential from weaker acids such as the carboxylic group (-COOH) attached to a solid support. The cation (Y^+) would exchange with other cations in the mobile phase, again depending on the relative concentration and affinity of the cations for the sulfonic acid group (51, 62, 66, 171, 197).

In HPLC ion-exchange chromatography, packings are based on two types of support material: polymeric resins and porous silica gel. Microparticulate silica ion-exchange resins are now generally preferred to the pellicular resins at least for chromatographic separation of organic anions or cations (62). Various cation-exchange resins differ structurally from each other in their hydrocarbon skeletons or acidic functional groups. The same cross-linked polystyrene polymer can be used with a variety of functional groups in addition to the sulfonic acid group itself. Wheals stated that besides aromatic sulfonic acids which were widely established in ion-exchange chromatography, alkanesulfonic acids have also been used (197).

The acid group can also be attached to silica gel via silane bonding, but according to Genieser et al , the alkyl spacer length is as a rule unknown, and the structures appearing in the literature are more or less based on speculations or hints from the different manufacturers (66). The exact structure of commercially available substituted silicas are not well understood.

1.6.2 Detectors

HPLC detectors have been reviewed extensively . In HPLC, the physical properties of the sample and mobile phase are often very similar. This has led to the development of two basic types of detectors (74) involving,

a) the differential measurement of a property common to both the sample and the mobile phase (bulk property); and

b) the measurement of a property that is specific to the sample (solute property). Examples of these two type detectors are refractive index (RI) and ultraviolet (UV) detectors which were used in this study.

1.6.2.1 Ultraviolet (UV) Detectors

The UV detectors are the most widely used of HPLC detectors (182). The principal of UV detectors is absorbance of electromagnetic radiation of ultraviolet light between the limits of 190 to about 320 nm. The fundamental law under which UV detectors operate is the Beer-Lambert law, as follows:

$$A = \epsilon BC$$

A is the absorbance at concentration, C, the a molar absorptivity, in a cell of length B. ϵ is the constant relating absorbance and concentration. The application is restricted to substances having π-bonding electrons and also to some extent, to those with unshared electrons. The detector cell usually consists of a short length of tubing, which is made to carry the eluent from the column and through which a beam of UV light is focussed onto a photoelectric cell. When solutes are present in the mobile phase, light is absorbed and thus the intensity of light falling on the photocell is reduced producing an electrical output which can be amplified and recorded by a recorder. At the present time, there are three types of UV detectors for HPLC analysis.

Fixed - Wavelength Detectors - Since the radiation source used in many UV detectors is a low pressure mercury vapour lamp, the predominant line in the spectrum is at 253.7nm, and other lines are filtered out to give monochromatic light at this wavelength. Manufacturers have

been able to utilize this 254 nm line to provide extremely stable, high sensitive detectors. The 254 nm fixed wavelength detectors have been described as the most low-cost and useful detectors (211).

Variable Wavelength Detectors - Any wavelength from 190 to 800 nm (UV and visible wavelengths) may be used. The individual sample components have high absorptivity at different wavelengths and thus, operation at a single wavelength would reduce the system's sensitivity or would even make the detection of certain sample components impossible. With this detector any wavelength in the ultraviolet and visible region can be monitored. The instrument is usually fitted with a deuterium lamp for UV wavelengths and a tungsten lamp for visible wavelengths. Individual wavelengths can be obtained with the use of a monochromator, and then passed through the sample to a photomultiplier. Finally, stopflow operation combined with peak scanning can be achieved with some variable wavelength detectors.

Diode Array UV Detectors - At least two companies now offer commercial diode array HPLC UV detectors. These detectors operate by passing the entire UV spectrum from a deuterium source (190-370nm) through a sample. The light is then dispersed through a holographic diffraction grating onto a photodiode array (at least one diode for each nm or more). If this detector is hooked to a data handling computer, one can get instant (1 second) spectra, 3-dimensional chromatograms, (absorbance verses time and wavelength) and enhanced signals by integrating the total sample absorbance.

Bakalyar and Henry studied the effects of flow rate and solvent composition on the variance of the response for an HPLC equipped with an UV detector and reported that the peak area was flow rate dependent. A 1% decrease in flow rate caused a 1% increase in peak area, while peak height measurements were much less flow rate dependent (14).

1.6.2.2 Refractive Index (RI) Detectors

The refractive index detectors are ranked second to the UV detectors in HPLC application (128). The principal of RI detectors is based on the measurement of the change in refractive index as the eluting sample enters the mobile phase. Thus, it is obvious that the

greater the refractive index difference between the eluted sample and the mobile phase, the larger the response. Thus the highest sensitivity in refractive index detection is obtained when there is the largest difference between sample and mobile phase. Because the only requirement in detection is that there is a difference in refractive index between sample and the mobile phase (this can be as little as 10^{-7} refractive index units, or 1 ppm of sample in the eluent)[46], RI detectors are sometimes considered as the universal detector in HPLC. In comparison of sensitivity, however, RI is about 1000 times less sensitive than UV detection. In addition, the RI detector is sensitive to change in the mobile phase composition, and is impractical with gradient elution. Change in temperature also causes a change in refractive index and, ideally the detector requires an environment stable to between 0.001 to 0.01°C (129). This is achieved by circulating a liquid (such as water or ethylene glycol) from a temperature regulated supply through a metal block which contains the refractometer. There are two basic types of RI detectors in common use today, deflection and Fresnel (or reflective) detectors. Both require the use of a two-path cell where the sample-containing side is constantly compared with the non-sample-containing reference side.

Deflection Refractometer - This is based on the deflection principle of refractometry. As eluting sample moves through the system, the change in composition of the sample path occurs relatively to the reference side. When no sample is present in the cell, the light passing through both sides is focussed. The changing angle of refraction moves the light as the sample is eluted through sample cell and an electrical signal is generated, with the degree of deflection proportional to the concentration of the sample(155).

Fresnel Refractometer - The Fresnel refractometer is based on the Fresnel principal, where refractive index changes between the mobile phase and the reference cell are monitored by the amount of light reflected at the cell-mobile-phase interface. The amount of light reflected is inversely proportional to the refractive index. The detector cell is made from a scored steel base plate, a Teflon spacer and a prism(155).

1.6.2.3 Some Conditions in the Detection

Detector Response - It is stated that the response for a given detector is different for different solutes; in case of the UV detector, the response is a function of the extinction coefficient (or molar absorptivity) of the solute, and for a refractive index detector, the refractive index of the solute (160). For this reason, (according to Beer's law, for example in UV detectors) the response of two detectors of the same type and geometry can only be compared if the same solute and mobile phase are employed.

Detector Sensitivity - Sensitivity is one of the most important parameters of an HPLC detector. Detector sensitivity is the minimum mass per unit time or minimum concentration of solute in mass per unit volume passing through the detector that can be discerned from the noise (160). It is generally accepted that a signal to noise ratio of 2 will permit unambiguous identification of a signal(160, 171).

2. EXPERIMENTAL

2.1 EQUIPMENT

2.1.1 HPLC System

Pump - A Beckman model 110A pump fitted with a Rheodyne model 7125, 50 μl loop injector was connected to an Altex model 420 flow programmer. The pressure during operation was found to be between 300 to 2000 psi.

Columns - Analytical columns were Whatman 25cm x 4.6mm i.d. Partisil 10, strong anion exchange (SAX) and strong cation exchange (SCX) columns which were protected by a 7cm x 2.1mm i.d guard column containing the same pellicular ion-exchanger as the analytical column. In addition, a pre-injector 25cm x 4.6mm i.d precolumn (Solvecon) containing Silica gel was attached.

Mobile Phase - The mobile phase consisted of potassium dihydrogen phosphate (KH_2PO_4) HPLC grade, prepared fresh daily, dissolved in HPLC water. Buffer concentration and pH were adjusted as detailed in the following chapters. The buffer was filtered through Millipore paper (0.45 μm) and degassed prior to use.

Detectors - A Laboratory Data Control Spectro-Monitor III Ultra Violet (UV) detector, model 1204A was used for the detection of nucleic acid compounds with the wavelength set at 254 nm. A Waters model R401 Differential Refractometer (RI) detector maintained at 25°C with a constant temperature ethylene glycol bath was used for the detection of the compounds other than nucleic acid substances.

Recorders - A Hewlett Packard, model 3388A Integrator Terminal was attached to the UV detector with a Terochem strip chart recorder attached for RI detector. The diagram of HPLC system is shown in Figure 2.1.

2.1.2 Spectrophotometer

A Beckman DU-8 UV-Visible Spectrometer was used for scanning of the absorbance spectrum of the compounds analysed.

2.1.3 Evaporator

A Buchi Rotavapor-R with water bath maintained at less than 50°C was used to concentrate samples.

2.1.4 Retort

A Reid Boilers Works Vertical Retort was used for the canning experiments. The temperature was set at 250°F(121°C). A chart recording thermometer was used to provide a permanent record of time and temperature of the cans processed. The recording thermometer was adjusted to agree with a laboratory mercury-in-glass thermometer. The diagram of the retort is given in the Figure 2.2.

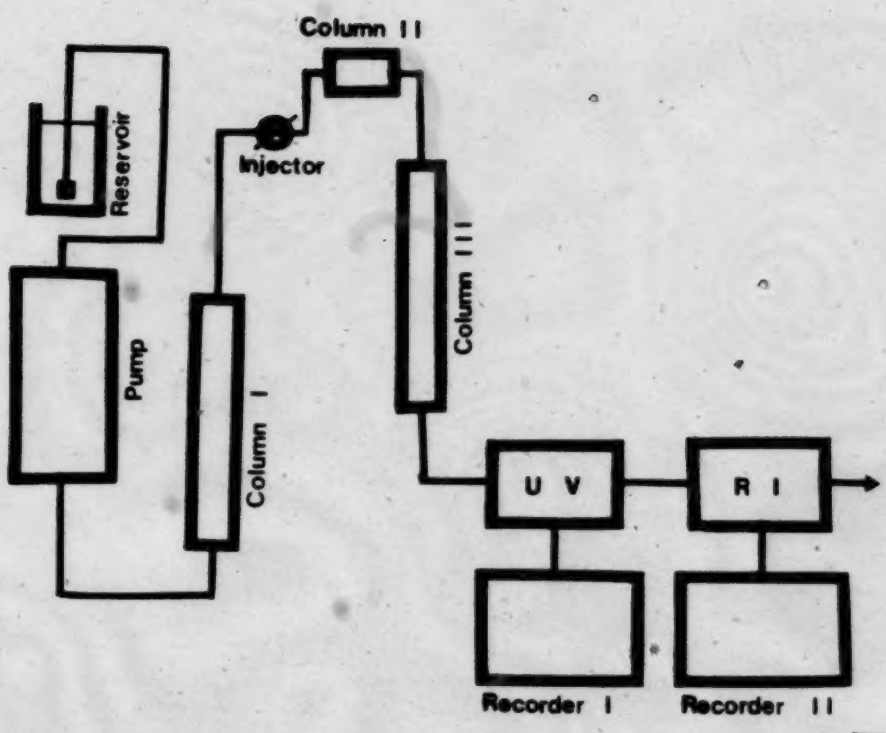


Fig. 2.1. Schematic Diagram of the HPLC System used for Flavor Enhancers Analysis

1. Drain Valves
2. Exhaust Valves
3. Hot Water
4. Pet Cock
5. Pump
6. Air Valve
7. Air Condenser
8. Automatic Steam Valve
9. Controller Timer
10. Power Switch
11. Start Button
12. Red Light
13. Air Valve to Retort for Over Pressure
14. Cold Water
15. Water Level Indicator
16. Constant Level Device
17. Thermocouple Exit
18. Pressure Gauge
19. Pressure Release Valve
20. Exhaust Timer
21. Control Element
22. Thermometer
23. Steam Spreader
24. Pressure Regulators

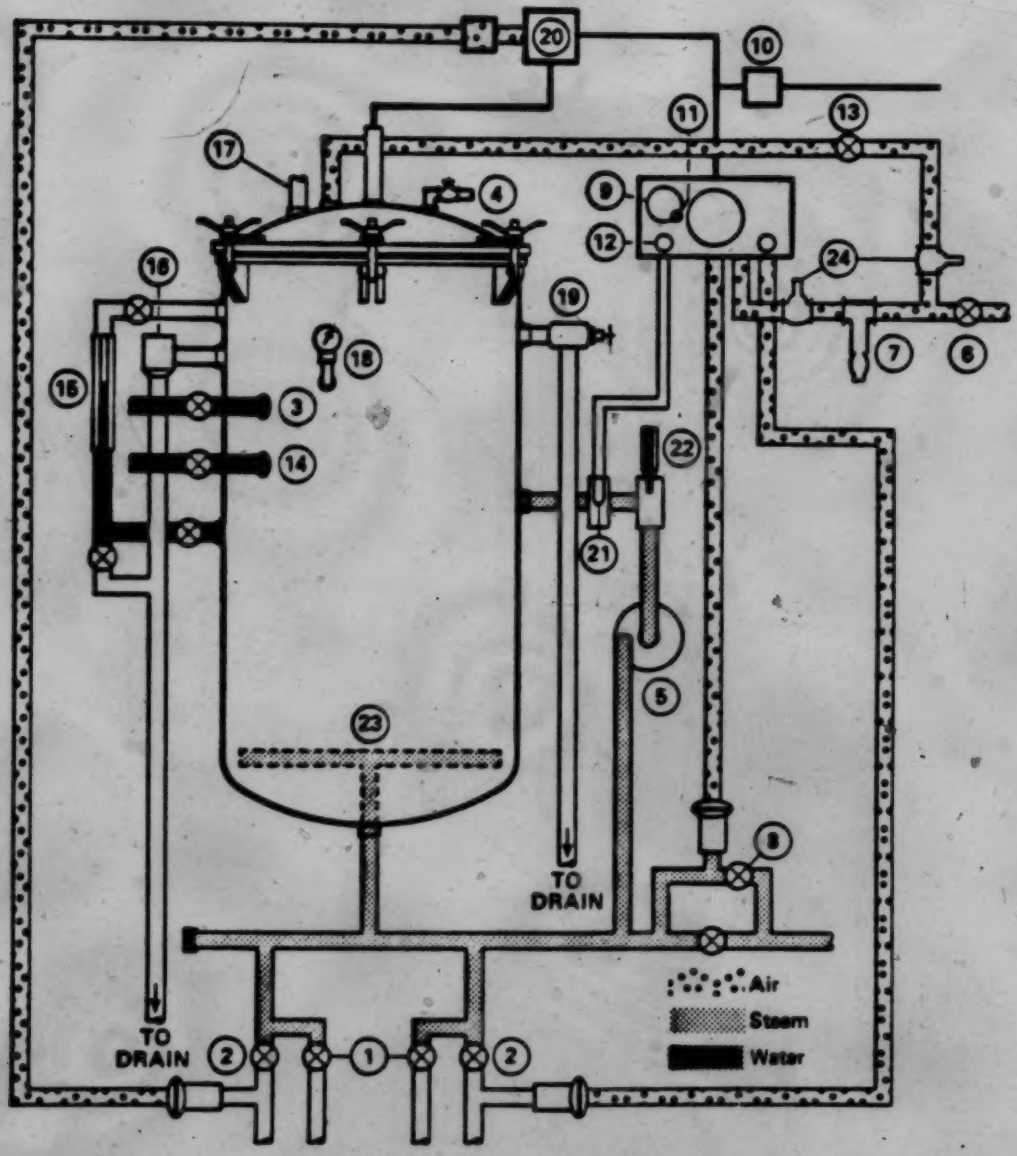


Fig. 2.2. The Automatic Steam Vertical Retort.

2.2 Reagents

HPLC grade water was prepared using a Millipore Milli-Q system (Millipore Corp. Bedford, MA. USA).

Celite (Celite 545) and potassium dihydrogen phosphate HPLC grade were obtained from Fisher Scientific. All other solvents and reagents were analytical grade or better.

Standards were obtained as follows : L-glutamic acid, from Calbiochem. Behring Corp. USA; D,L-pyroglutamic acid, from Puriss Koch-Light Laboratory UK; monosodium glutamate (MSG), from BDH Biochemical U.K.; sodium chloride, from Fisher Scientific; guanine, from Aldrich Chemical, USA; glucose, guanosine and inosine, from Terochem, Canada; and hypoxanthine, inosine-5'-monophosphate disodium salt, guanosine-5'-monophosphate disodium salt, L-aspartic acid, casein and potato starch all were obtained from Sigma, Illinois, USA.

2.3 HPLC Analysis of Foods

2.3.1 Samples

Rice soup (rice : water in the ratio of 1:10) was prepared in the laboratory with no flavor enhancers added. Commercial food products consisting of canned foods, instant noodles, instant soups, beef in-a-Mug, chicken in-a-Mug, soy sauce (tempura type) and potato chips were purchased from the grocery stores, located in the Edmonton area. The restaurant soups were purchased from chinese restaurants located in Edmonton and Calgary, Alberta. Japanese miso soup was supplied by a Japanese restaurant located in Edmonton, Alberta.

2.3.2 Conditions of HPLC

The analysis was carried out using a SAX column along with the mobile phase of 0.017M potassium dihydrogen phosphate buffer, pH adjusted to 4.0 with diluted phosphoric acid, and a flow rate of 1.0 ml/min. The detection system consisted of UV and RI detectors

(Figure 2.1).

2.3.3 Sample Preparation

Canned samples were weighed before and after emptying to determine the total can contents. Both liquid (soup) and canned products were then homogenized in a Waring blender before analysis. Dried products were ground to a powder with a motar and pestle. For the analysis, 10 g of wet or 1 g of dry sample were used. The sample was weighed to an accuracy of ± 0.1 mg.

2.3.4 Extraction Procedure

The extraction procedure used was a modification of the standard procedure of the Association of Official Analytical Chemists (AOAC) for analysis of glutamate(139) originally developed by Fernandez-Flores et al (54). The prepared sample was added to a 150 ml beaker containing 30 ml of buffer and a magnetic stir bar. The contents of the beaker were stirred for 15 minutes and then 40 ml acetone was added. After an additional 5 minutes stirring, the beaker contents were left standing for about 5 minutes, and filtered through a Celite pad, that had been washed with 20 ml of 50% acetone in water. The beaker and residue on the Celite pad were washed with additional 50% acetone in water (6 x 20ml). The filtrate was transferred to a round bottom flask and evaporated to about 10ml and then transferred to a 50ml volumetric flask. The round bottom flask was rinsed several times with water, the rinsings and additional water were used to bring the volume to 50 ml. Samples were filtered through Millipore paper (0.45 μm) prior to injection into the HPLC system. The flow diagram of this procedure is given in Figure 2.3.

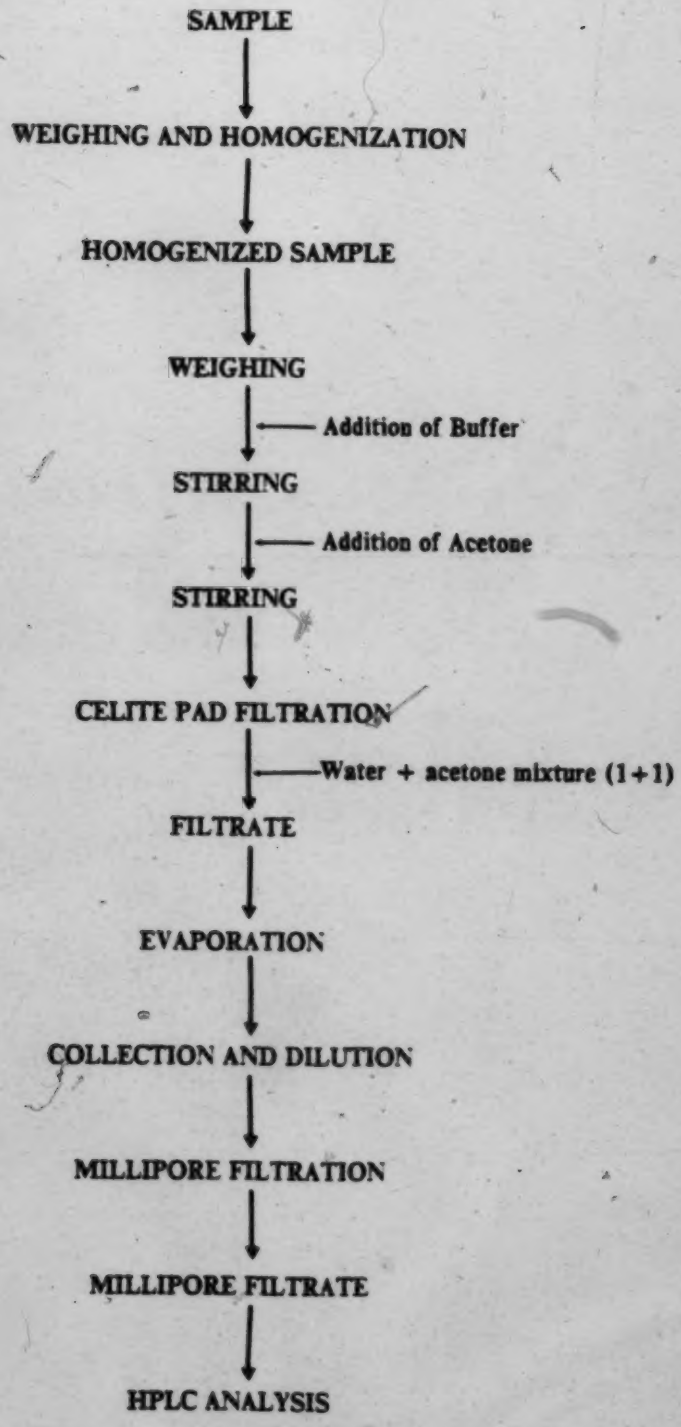


Fig. 2.3 Flow Diagram of Flavor Enhancers Extraction Procedure for HPLC Analysis.

2.3.5 Standard Solution Preparation

A standard mixture containing L-glutamic acid, IMP, GMP, pyroglutamic acid, L-aspartic acid and sodium chloride was prepared fresh daily. The concentration of the standard compounds in solution was maintained close to the concentration of each compound contained in the sample solution.

2.3.6 Identification and Quantitation

The peaks from the chromatogram were identified by retention time and sometimes checked by "spiking" the sample. The "spiking" involved addition of a known standard of the compound of interest to the sample and observing which of the peaks in the sample chromatogram increased.

The external standard method was used for the quantitation using peak area comparison to the averaged area of the standard injected before and after the sample. A minimum of two injections for each sample were carried out. The peak areas and the concentration of the compounds analysed were calculated using the following equations:

$$PA = PH \times W$$

$$CONC.(Sam) = [PA(Sam) / PA(Std)] \times [CONC(Std)]$$, where,

PA = peak area

PH = peak height

W = peak width at half of peak height

(Sam) = Sample

(Std) = Standard

CONC = Concentration in μg/ml for nucleotide compounds and mg/ml for the non-nucleotide compounds.

MSG was then converted from glutamic acid by the factor of 1.15, or

Amount of MSG = 1.15 x amount of glutamic acid.

2.4 Preliminary Canning Experiment

2.4.1 Formulation of Experimental Canned Soup

The ingredients of the "soup" prepared for this experiment consisted of protein, sugar, starch, salt and flavor enhancers. It was prepared according to the formulation of "Beef Flavored Soup" suggested by Ajinomoto Co. Inc. Japan as shown in Table 2.1, with the amount of sugar, protein and starch used in this study equivalent to the ingredients suggested, as shown in the Table 2.2. The amount of flavor enhancers added to the experimental soup simulated the situation in the canning industry which can be found in section 2.3 .

2.4.2 Sample Preparation

2.4.2.1 Preparation of Standard Solution

A standard solution containing 54mg/ml MSG, 1080 μg/ml disodium IMP and 1080 μg/ml disodium GMP was prepared fresh before canning. This solution was kept in a glass container, closed tightly and used as a stock standard solution during canning processing and the later analysis procedure. The solution was chromatographically analysed prior to use for canning.

2.4.2.2 Preparation of Buffer Solution

0.01M phosphate buffer of pH 5.50 was prepared by dissolving 2.7218g potassium dihydrogen phosphate in about 1800ml HPLC water, adjusting the pH to 5.50 with 1.0M potassium hydroxide solution, transferring to a 2000 ml volumetric flask and making up to volume with water.

TABLE 2.1

Formulation of Beef Flavored Soup

Ingredients	Amount (g)
Beef extract	0.200
Beef fat	0.550
Vegetable mixture*	0.215
Spices, herbs mixture**	0.046
Salt	0.800
Caramel	0.020
IMP	0.012
MSG	0.140
Water	100.000

* Carrot powder 0.10g, onion powder 0.10g, celery powder 0.015g

** White pepper 0.010g, black pepper 0.010g, garlic powder 0.015g laurel 0.001g, parsley 0.010g.

Source from Ref. 1 .

TABLE 2.2

Formulation of Standard Soup Prepared in the Laboratory.

Ingredients	Amounts (%)
MSG	0.150
IMP	0.003
GMP	0.003
Protein (Casein)	1.200
Sugar (Glucose)	0.500
Starch	1.000
Salt (NaCl)	0.800
Buffer	96.344

2.4.2.3 Examination of Protein and Starch

20g of Casein or starch was extracted by dissolving the sample in water, stirring for about 30 min on a magnetic stirrer and centrifuging for 30 min at 10,000 rpm. The supernatant was transferred to a round bottom flask, evaporated to a small volume, transferred to a 25 ml volumetric flask and made up to volume with water.

The solution of protein was then filtered through Millipore paper and analysed chromatographically.

The solution of starch was examined using the Munson and Walker method(141) for sugar determination.

2.4.3 Canning Procedure

Five types of "soup" were prepared as following.

Group 1 : Cans containing the ingredients listed in Table 2.2, dissolved in buffer.

Group 2 : Cans containing the ingredients listed in Table 2.2, without sugar, dissolved in buffer.

Group 3 : Cans containing the ingredients listed in Table 2.2 without protein, dissolved in buffer.

Group 4 : Cans containing the ingredients listed in Table 2.2, without starch, dissolved in buffer.

Group 5 : Cans containing the ingredients listed in Table 2.2 without flavor enhancers, dissolved in buffer, used as a sample blank.

The required ingredients for each group were weighed accurately according to the ratio listed in Table 2.2. Starch was added to a 1000 ml beaker containing about 700 ml buffer, stirred and gently heated on a hot plate stirrer until it dissolved completely. The starch solution was cooled to about 40°C and other ingredients were then added. Protein was dissolved in buffer prior to addition to the starch solution. The beaker contents were stirred again on a magnetic stirrer without heating until everything had dissolved. The weight of the beaker

contents was then adjusted to 1000g by adding buffer. The pH of solution was recorded.

Four lacquered tin-plated cans 6.7cm x 5.2 cm i.d. (capacity 140ml) were used and 125g of "soup" solution was placed in each can. Stock standard solution of flavor enhancers was added to two cans (3.5ml in each can), these cans were named "test cans". Two other cans were prepared with no added flavor enhancers and used for controls which were termed as "control cans". The list of can numbers is given in Table 2.3.

Next, all cans were exhausted using steam for 5 min. Sealed cans were then processed in a pilot plant retort. The retorting temperature was maintained at 250°F for 30 min. At the completion of the retorting period, cans were cooled and stored in a walk-in cooler maintained at 4°C for further analysis.

The flow diagram of canning procedure is given in Figure 2.4.

2.4.4 Sample Analysis

2.4.4.1 Method

Test cans and control cans in each group were analysed simultaneously using the HPLC method, given in section 2.3 .

2.4.4.2 Extraction Procedure

The can was opened just before analysis, the pH of the "soup" was measured and the "soup" color observed and recorded. The "soup" was then transferred to a 250 ml beaker and the can was rinsed several times with water. The weight of the "soup" was then adjusted to 200g using water. In case of the control cans, 3.5 ml flavor enhancers stock solution was added prior to adjusting the weight to 200g. Extraction was carried out in duplicate. The isolation procedure for the flavor enhancer followed the chart diagram given in Figure 2.3.

TABLE 2.3

The List of Can Numbers for Prepared "Soup"

Soup group	Sample can numbers		Control can numbers	
	1st can	2nd can	1st can	2nd can
1	1 - T* - 1	1 - T - 2	1 - C* - 1	1 - C - 2
2	2 - T - 1	2 - T - 2	2 - C - 1	2 - C - 2
3	3 - T - 1	3 - T - 2	3 - C - 1	3 - C - 2
4	4 - T - 1	4 - T - 2	4 - C - 1	4 - C - 2
5	5 - T - 1	5 - T - 2	5 - C - 1	5 - C - 2

* T=Test Can, C=Control Can.

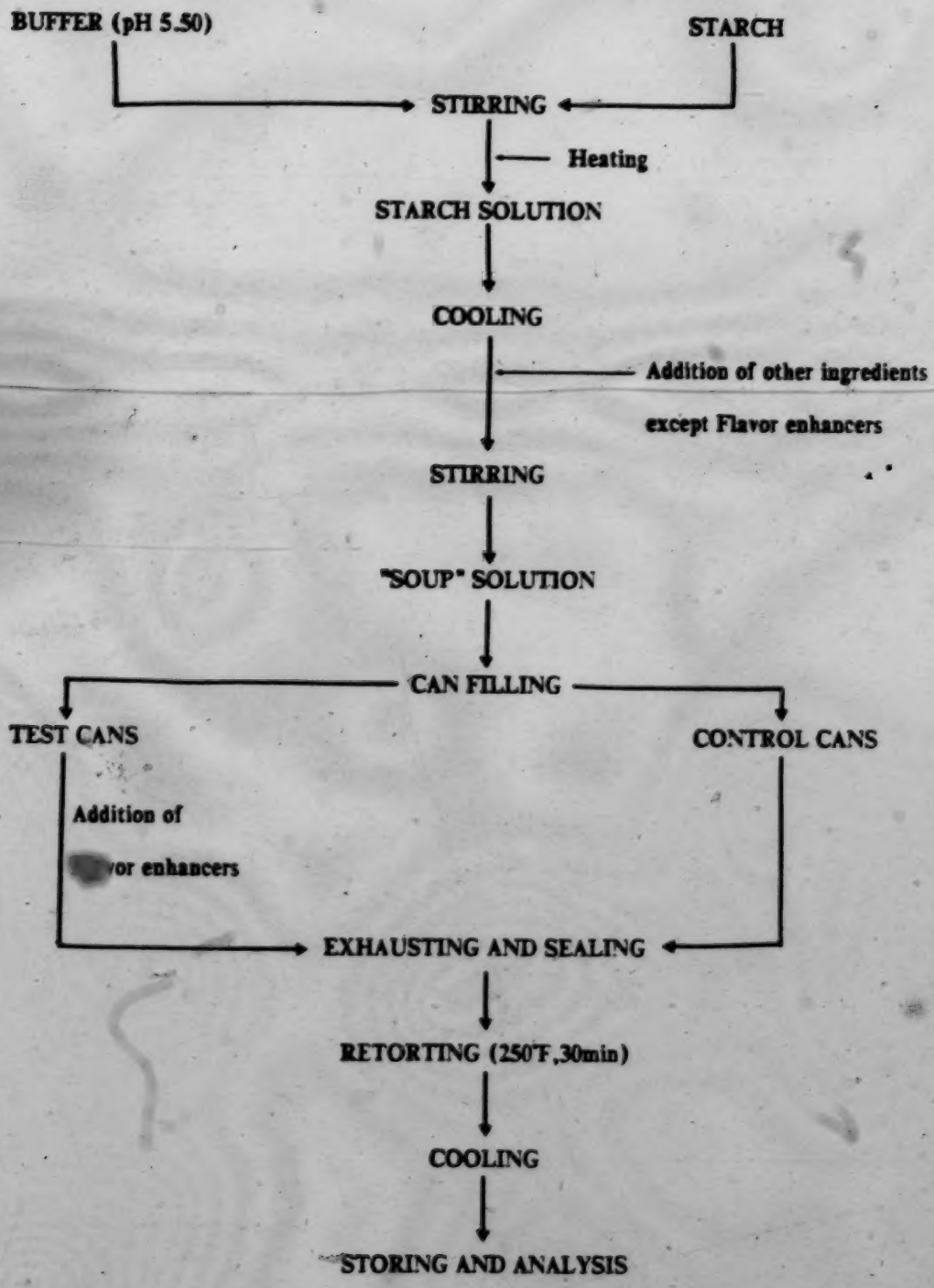


Fig. 2.4 Flow Diagram of Canning Procedure of Preliminary Canning Experiment.

2.4.4.3 Quantitation and Recovery Calculation

The following equations were used for the recovery calculations.

$$A = CxD/ExF \text{ (mg/ml)}$$

$$\text{Amount Found} = \{\text{Peak Area (B)/Peak Area (A)}\} \times \{AxExF/D\} \text{ (mg)}$$

$$\% \text{ Recovery} = \{\text{Amount Found/C}\} \times 100$$

where,

A = concentration of a compound present in the injected solution of control sample in mg/ml.

B = concentration of a compound present in the injected solution of test sample in mg/ml

C = amount of flavor enhancer added as follows:

C(MSG) = 189 mg, C (IMP) = C (GMP) = 3.780 mg.

D = weight of soup taken for analysis in gram.

E = total weight of homogenized "soup" in gram.

F = total volume of Millipore filtrate in ml.

When, D,E,F values were kept constant in every analysis, the calculation of percentage recovery was simplified as shown in the following equation.

$$\% \text{ Recovery} = \{\text{Peak Area (B)/Peak Area (A)}\} \times 100$$

2.5 Canning of Flavor Nucleotides

2.5.1 Sample Preparation

2.5.1.1 Preparation of Standard Solution

A standard mixture solution containing equal concentration of disodium IMP and disodium GMP at 12.75mg/ml was prepared with water and used as a stock standard solution. The standard solution was analysed chromatographically prior to use for canning.

2.5.1.2 Preparation of Buffer Solution

0.02M phosphate buffer was prepared by dissolving 5.4436g potassium dihydrogen phosphate in about 1800 ml HPLC water, adjusted pH to 4.50, 5.50 and 6.50 with diluted phosphoric acid or 1.0M potassium hydroxide solution, transferred to a 2000 ml volumetric flask and made up to volume with water.

2.5.2 Canning Procedure

Eight lacquered tin-plated cans 10.9cm x 7.4cm i.d (capacity 470ml) were used for each different pH buffer group. 424g of buffer was weighted into each can, with 1 ml of stock standard solution added. Two cans in each group were sealed and kept without heating and used as controls. Another six cans were exhausted using steam for 5 min., sealed and divided in three sections of different retorting time of 15, 30 and 60 min. The retorting temperature was maintained at 250°F. Six thermocouples were placed into cans containing buffer which were used to monitor the temperature during the retorting period. The can numbers are listed in Table 2.4 and the flow diagram of canning procedure is given in Figure 2.5.

2.5.3 Sample Analysis

2.5.3.1 Method

Test cans and control cans were analysed simultaneously using the HPLC method. Nucleotides, and the decomposition compounds from nucleotides were analysed using SAX and SCX columns, respectively. The concentration, pH and flow rate of buffer in the HPLC mobile phase were as follows:

0.017M, pH 4.00, flow rate 1.0ml/min for the analysis of nucleotides,

0.01M, pH 3.20, flow rate 0.5ml/min for the analysis of nucleosides,

0.01M, pH 3.60, flow rate 0.5ml/min for the analysis of bases.

The can was shaken prior to opening and the solution injected directly into the HPLC system. The analyses were all carried out within one week. As soon as a can was opened the

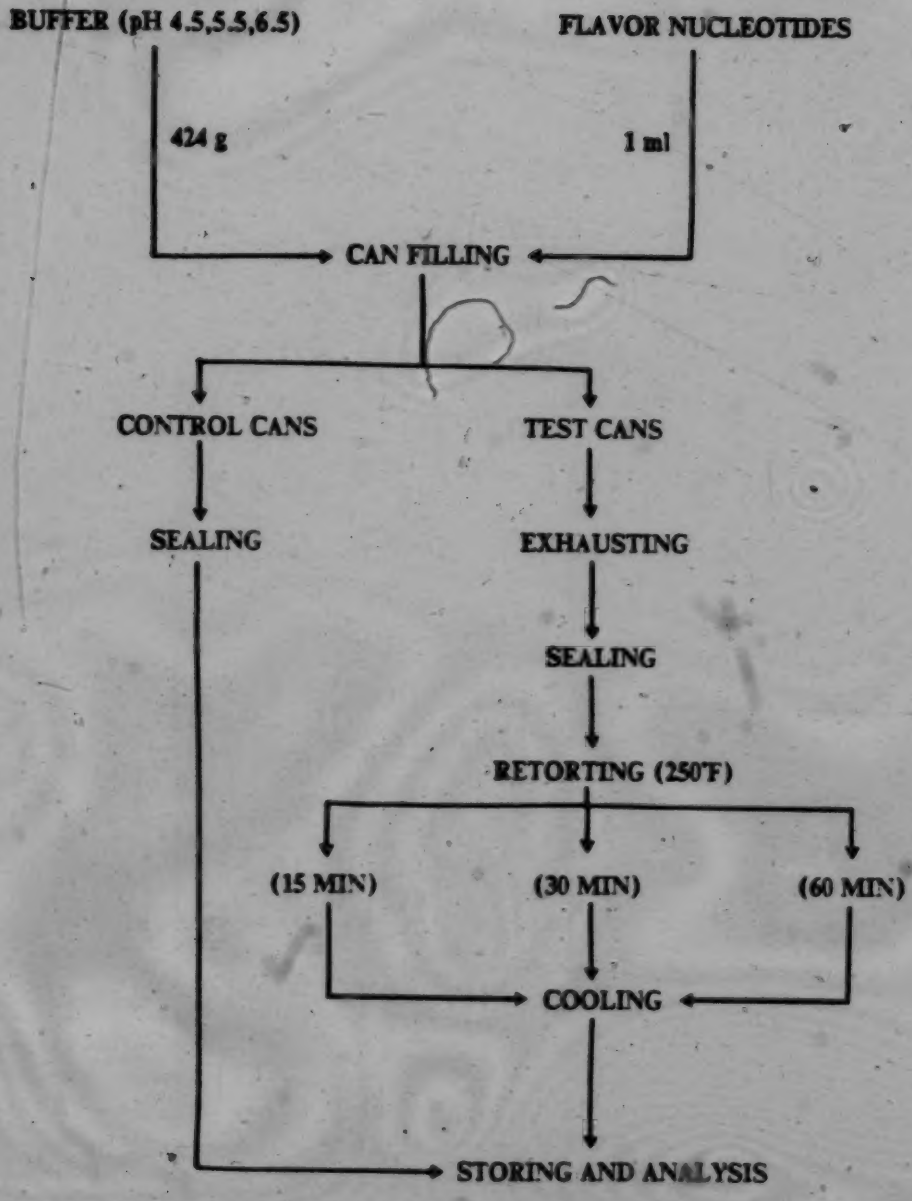


Fig. 2.5 Flow Diagram of Canning Procedure for Flavor Nucleotides.

TABLE 2.4

The Numbering of Flavor Nucleotides Cans

pH	Retorting time	Can numbers *
4.50	0	0 - 45 - C1; 0 - 45 - C2
	15	15 - 45 - T1; 15 - 45 - T2
	30	30 - 45 - T1; 30 - 45 - T2
	60	60 - 45 - T1; 60 - 45 - T2
5.50	0	0 - 55 - C1; 0 - 55 - C2
	15	15 - 55 - T1; 15 - 55 - T2
	30	30 - 55 - T1; 30 - 55 - T2
	60	60 - 55 - T1; 60 - 55 - T2
6.50	0	0 - 65 - C1; 0 - 65 - C2
	15	15 - 65 - T1; 15 - 65 - T2
	30	30 - 65 - T1; 30 - 65 - T2
	60	60 - 65 - T1; 60 - 65 - T2

* 1st number = Retorting time; 2nd number = pH x 10; 3rd number = Can ordinal number; C = Control can; T = Test can.

analysis for all compounds was completed within the day.

2.5.3.2 Preparation of Standard Solution

The HPLC standard solutions were prepared fresh before injection into the HPLC. The concentration of compounds in the standard solution were maintained close to the concentration of the compound present in the analysed solution. IMP and GMP standard solution was prepared from the stock standard solutions (used for canning). Inosine and hypoxanthine standard solution was prepared by dissolving the compounds in 0.01 M phosphate buffer at pH 3.20. Guanosine standard solution was prepared by dissolving the compound in a mixture of 0.1% (V/V) dimethylformamide (DMF) in water. Guanine standard solution was prepared by dissolving the compound in 0.1N HCl solution.

2.5.3.3 Quantitation of Decomposition

The external standard method was used for quantitation using peak area comparison to the averaged peak area of the standard injected before and after the sample. A minimum of two injections were carried out. The concentration of the analysed compounds and the percent of nucleosides obtained from nucleotides were calculated from the following equations:

$$\text{CONC.(Sam)} = \{\text{PA (Sam)/PA (Std)}\} \times \{\text{CONC (Std)}\}$$

$$\% \text{ N} = \{\text{N (ob)/ N (a)}\} \times 100$$

where,

CONC (Sam) = Concentration of sample, in μg/ml,

CONC (Std) = Concentration of standard, in μg/ml,

PA (Sam) = peak area of sample,

PA (Std) = peak area of standard,

% N = percent of nucleoside,

N(ob) = amount of nucleoside obtained, in μM,

N(a) = amount of nucleotide added, in μM.

The amount of the compounds analysed was converted from μg/ml to μM using the following factors:

1 μg/ml (disodium IMP) = 2.550 μM (disodium IMP)

1 μg/ml (disodium GMP) = 2.456 μM (disodium GMP)

1 μg/ml (Inosine) = 3.728 μM (Inosine),

1 μg/ml (Guanosine) = 3.530 μM (Guanosine),

1 μg/ml (Hypoxanthine) = 7.347 μM (Hypoxanthine),

1 μg/ml (Guanine) = 6.617 μM (Guanine).

2.6 Canning of Flavor Nucleotides and their Nucleosides

2.6.1 Sample Preparation

2.6.1.1 Preparation of Standard Solutions

Four standard solutions of disodium IMP (12mg/ml in water), disodium GMP (12mg/ml in water), Inosine (8mg/ml in 0.1M KH_2PO_4 buffer) and Guanosine (4mg/ml in 0.1M HCl solution) were prepared. These solutions were analysed chromatographically prior to canning.

2.6.1.2 Prepartion of buffer and sample solution

Phosphate buffer (0.02 M) solution was prepared by dissolving 5.4436 g KH_2PO_4 in a 2000 ml beaker containing about 1900 ml HPLC water. The pH was adjusted to 4.5 and 9.0 using diluted phosphoric acid or 1.0M potassium hydroxide, 5 ml of the standard solution of IMP, GMP, Inosine and 10 ml of the standard solution of Guanosine were pipetted separately into six different 2000 ml volumetric flasks. Four buffer solutions of pH 4.5 and 9.0 were transferred to the 2000 ml volumetric flasks containing IMP and GMP. Another two buffer solutions of pH 4.5 were transferred to the 2000 ml volumetric flasks containing Inosine and Guanosine. Water was used to rinse the beaker and make the flasks up to volume. The pH 1.5

solution was prepared by dissolving 5.4436g KH_2PO_4 in a 2000 ml beaker with about 1800 ml HPLC water, adding about 93 ml phosphoric acid (85%) and then transferring a 2000 ml volumetric flask containing 5 ml standard solution of nucleotide compounds (IMP or GMP), and making up to volume with water. This solution was approximately equal to 0.70M total phosphate. Each volumetric flask was shaken to mix the test compound and the buffer.

2.6.2 Canning Procedure

Four lacquered tin-plated cans 10.9 cm x 7.4 cm i.d (capacity 470 ml) were used for each sample solution, 425.0g of sample solution was weighed into each can. Two cans in each group were sealed, kept without heating and used as the control of heating effects. Other cans were exhausted using steam for 5 min, sealed and retorted for 60 min, the retorting temperature was maintained at 250°F. Thermocouples were used to monitor temperature during the retorting period. The can numbers are listed in Table 2.5 and a flow diagram of the canning procedure is given in Figure 2.6.

2.6.3 Sample Analysis

The analytical method and the HPLC conditions were similar to section 2.5.3. Both addition and external standard methods were used for the samples quantitation at the lower pH (pH 1.5) and higher pH (pH 9.0). The samples at pH 4.5 were quantitated using the external standard method.

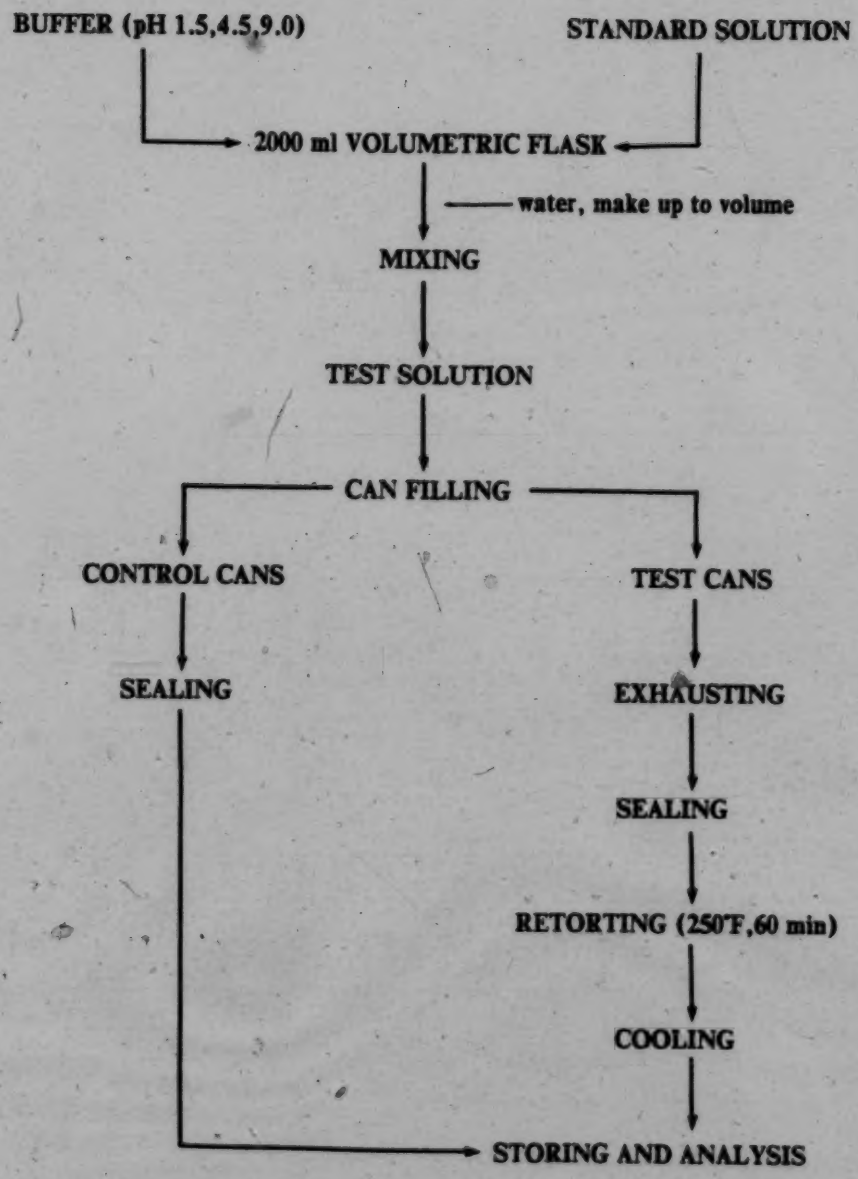


Fig. 2.6 Flow Diagram of Canning Procedure for Flavor Nucleotides andTheir Nucleosides Canned Individually.

TABLE 2.5

The Numbering of Flavor Nucleotides and Nucleosides Canned Individually.

pH	Compound	Can numbers •
1.5	GMP	G - 15 - C1; G - 15 - C2
		G - 15 - T1; G - 15 - T2
4.5	GMP	G - 45 - C1; G - 45 - C2
		G - 45 - T1; G - 45 - T2; G - 45 - T3
9.0	GMP	G - 90 - C1; G - 90 - C2
		G - 90 - T1; G - 90 - T2
1.5	IMP	I - 15 - C1; I - 15 - C2
		I - 15 - T1; I - 15 - T2
4.5	IMP	I - 45 - C1; I - 45 - C2
		I - 45 - T1; I - 45 - T2; I - 45 - T3
9.0	IMP	I - 90 - C1; I - 90 - C2
		I - 90 - T1; I - 90 - T2
4.5	Inosine	PSI - 45 - C1; PSI - 45 - C2
		PSI - 45 - T1; PSI - 45 - T2
4.5	Guanosine	PSG - 45 - C1; PSG - 45 - C2
		PSG - 45 - T1; PSG - 45 - T2

• 1st letter = Compound shortened name; 2nd letter : C = Control can, T = Test can. 1st Number = pH x 10; 2nd number = can ordinal number.

3. RESULTS AND DISCUSSION

3.1 Methodology

Two detectors were required for the analysis of flavor enhancers, since glutamate and salt (sodium chloride) were added to food in amounts that could be detected using a RI detector while the small quantities of the nucleotides (IMP and GMP) usually added to food could not be detected using the RI detector. Conversely, IMP and GMP could easily be detected using an UV detector while glutamate, aspartate and pyroglutamic acid did not have significant UV absorption in a region free of interference from other compounds. Hence, glutamate, aspartate, pyroglutamic acid and chloride were detected using a RI detector and the nucleotides were monitored at 254 nm using an UV detector. The wavelength of 254 nm was chosen as a compromise between the UV maximum of IMP and GMP (Figure 3.1) and is also a convenient wavelength for cheaper filter UV detectors.

The mobile phase conditions of buffer, concentration, pH, and flow rate were optimized so that gradient elution was not required for the separation of the six compounds studied (Figure 3.2). Buffer was prepared fresh daily, followed by Millipore filtration and degassing before use.

In order to observe the possible interference, various taste components of food and food preservatives were chromatographed and their retention times recorded and compared to that of the compounds studied. The acidic group examined consisted of: adenosine 5'-monophosphate (AMP), a natural savory compound less active than IMP and GMP (106), present in large amount in food (104); Xanthosine 5'-monophosphate (XMP), a poor flavor enhancer (106); the preservatives sorbic acid and benzoic acid and the organic acids which are commonly found in food including, cinamic acid, citric acid, tartaric acid, malic acid, succinic acid, lactic acid, and acetic acid. The amino acid group examined consisted of lysine, asparagine, β-alanine, phenylalanine, proline, tryptophan, 4-amino-butyric acid (GABA), and glutamine. The basic group examined consisted of hypoxanthine, inosine, guanine,

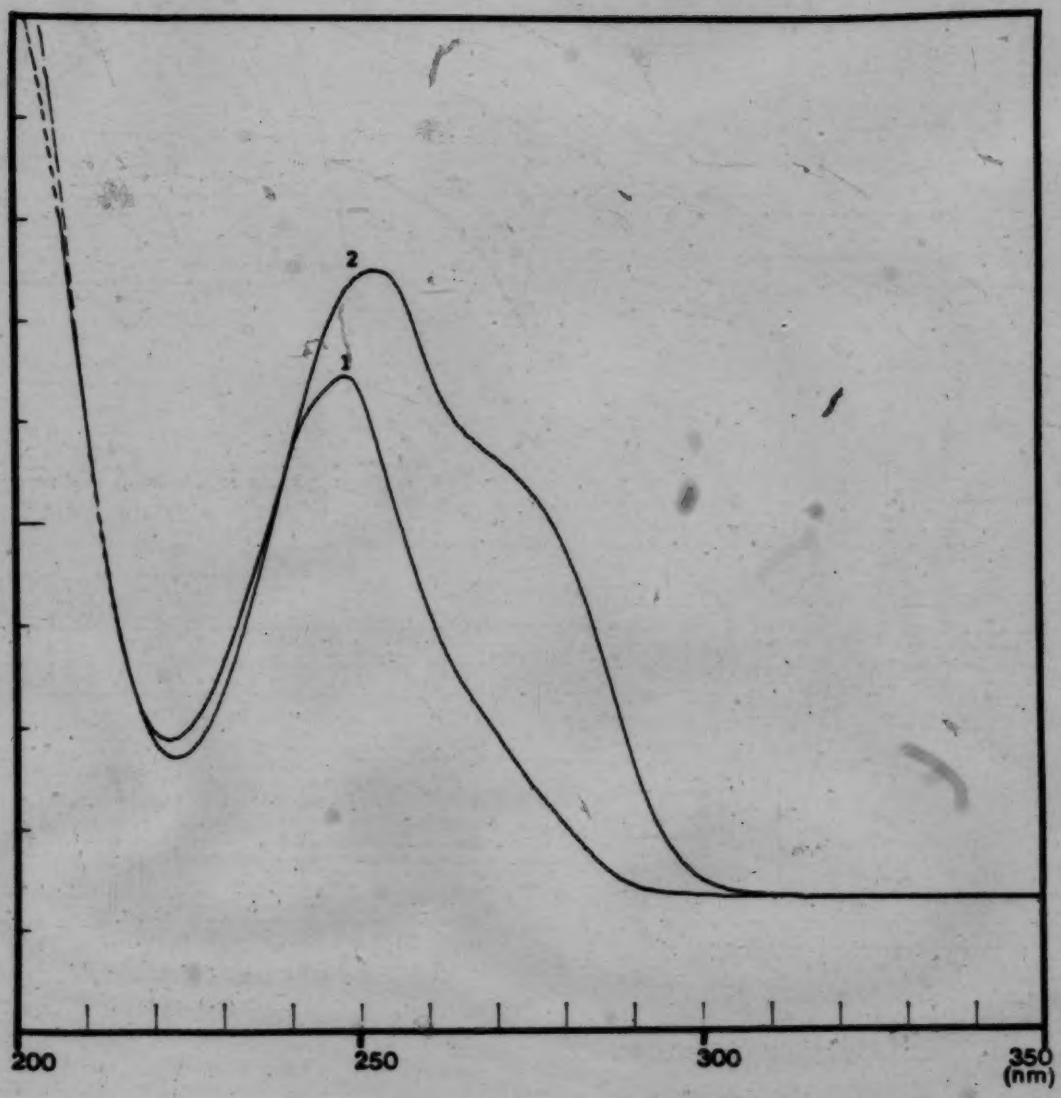


Fig. 3.1 Spectrophotometric Scans of Standard Compounds in the Phosphate Buffer pH4.0

1 = IMP (122.40 μM), 2 = GMP (117.88 μM).

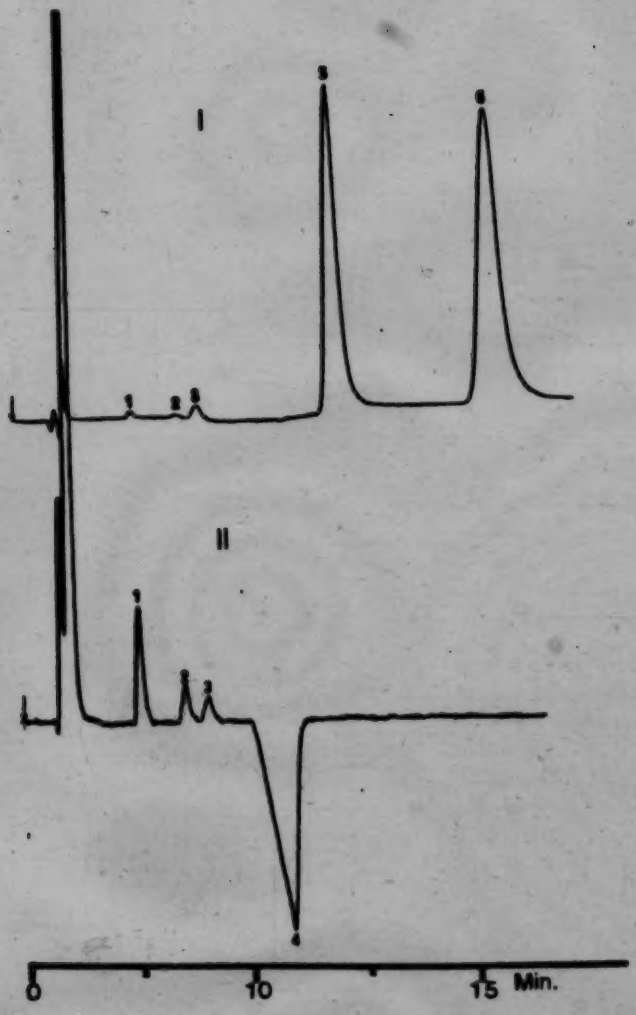


Fig. 3.2. Liquid Chromatogram from UV(I) and RI(II) of 1 : Glutamic acid (0.4mg/ml); 2 : Aspartic acid (0.2 mg/ml); 3 : Pyroglutamic acid (0.2 mg/ml); 4 : Chloride (3.0 mg NaCl/ml); 5 : IMP (8.0 μg/ml) and 6 : GMP (8.0 μg/ml) at a Flow Rate of 1 ml/min with 0.017 M pH 4.0 Phosphate Buffer.

and guanosine, which are the bases and nucleosides of IMP and GMP respectively, and had UV absorption similar to IMP and GMP (Figures 3.3 and 3.4). None of the above compounds interfered with the compounds of interest. The acidic compounds had different retention times from the compounds studied. The neutral and basic amino acids as well as the metabolites of IMP and GMP appeared at or near the solvent front in the HPLC system using the SAX column, as expected.

To further prove that interfering compounds were not co-eluting with IMP and GMP, quantitation of two samples containing nucleotides was carried out at various wavelengths (Table 3.1). The difference in amounts of IMP and GMP obtained was not significant with a wave length change of ± 15 nm.

Measurement of peak areas of different concentrations gave a linear response for all compounds studied (Table 3.2)

Two samples were analysed five times each to examine the precision of the extraction (Figure 2.3) and the analysis procedure. The result are shown in Tables 3.3 and 3.4.

Recovery studies were carried out on a consomme soup containing flavor enhancers and a rice soup without flavor enhancers. The results of these recovery studies are given in Tables 3.5 and 3.6.

It was found that liquid food samples such as consomme soup could be diluted, filtered and injected directly for analysis. It was felt, however, that this procedure would shorten the HPLC column life. Charcoal decolorization used with MSG and pyroglutamic acid analysis (173) was eliminated because of adsorption of nucleotides by charcoal. This elimination did not seem to detrimentally affect the analysis of other non-nucleotide compounds. If Celite was not pre-washed with water-acetone, it was found that HPLC detectable material (although non-interfering) was eluted from the celite.

It was illustrated that initial dilution of the sample in water(54) rather than buffer resulted in cloudy suspensions which caused filtration problems with non-salted foods, especially in samples containing large amount of starch. For this reason, it was essential to use

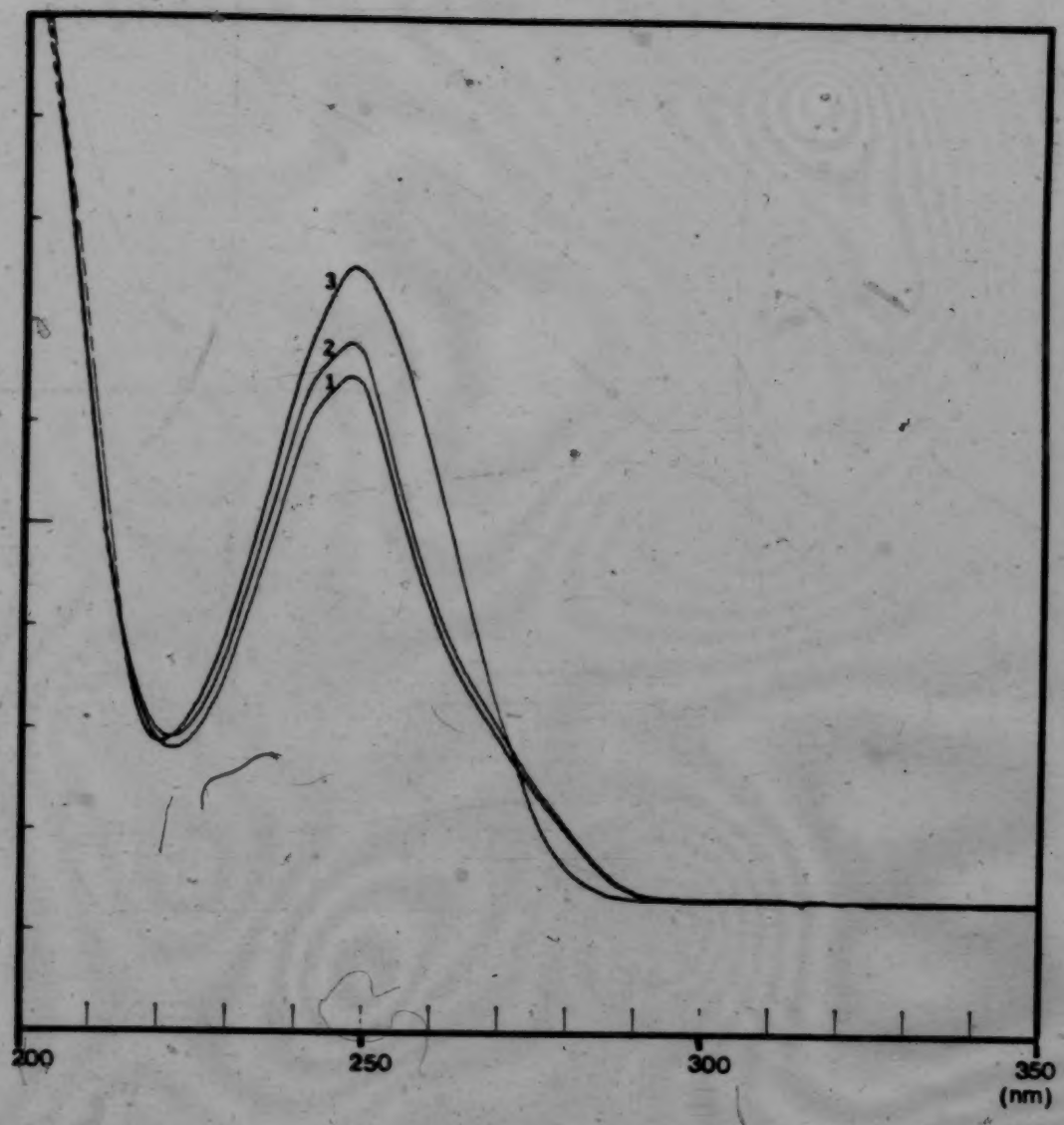


Fig. 3.3 Spectrophotometric Scans of Standard Compounds 1 = IMP (122.40 μM), 2 = Inosine (101.40 μM), Both in the Phosphate Buffer pH 4.0 and 3 = Hypoxanthine (146.94 μM) in 0.1N HCl

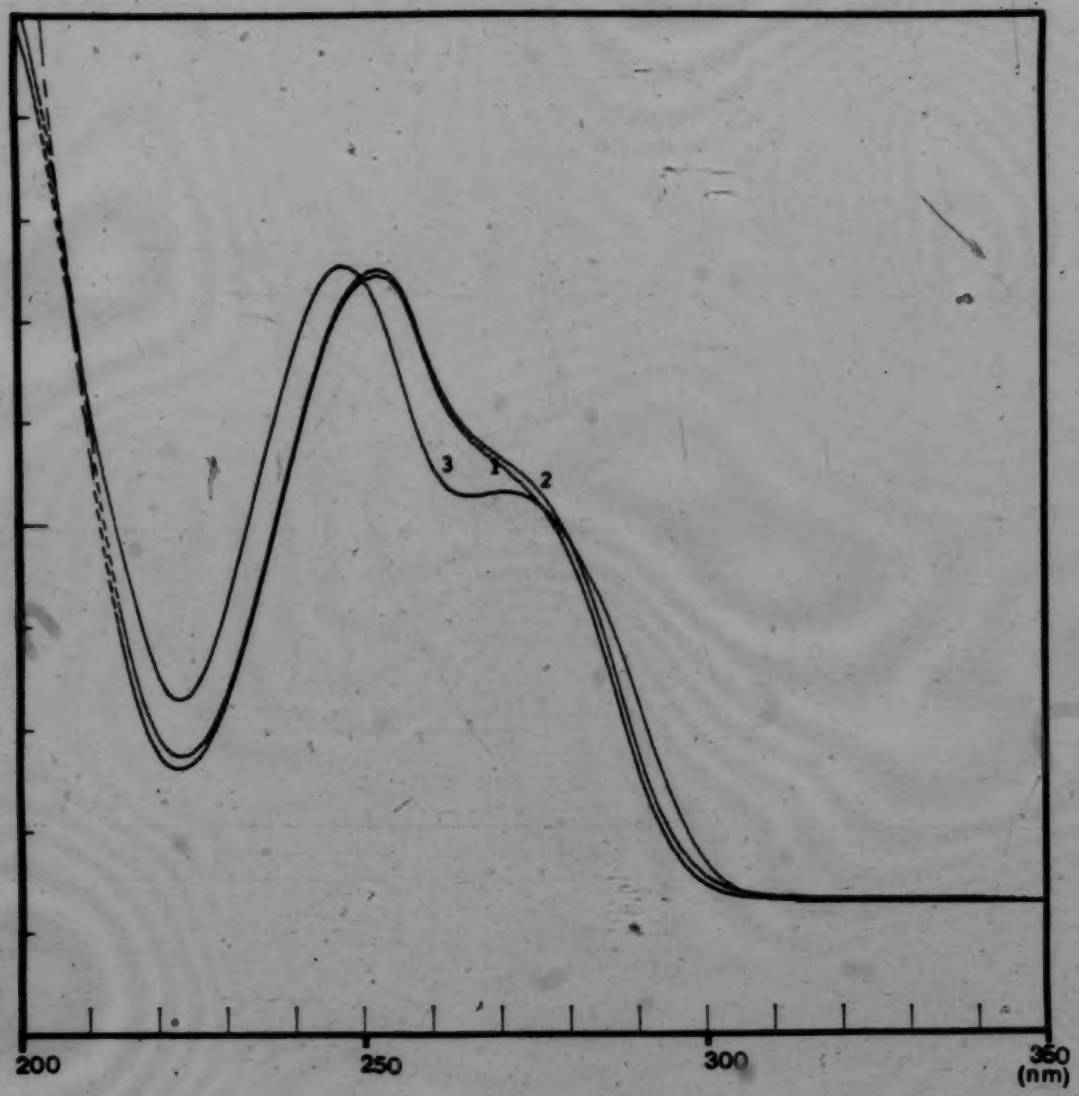


Fig. 3.4 Spectrophotometric Scans of Standard Compounds 1 = GMP (117.88 μM) in the Phosphate Buffer pH 4.0. 2 = Guanosine (112.96 μM) and 3 = Guanine (132.34 μM), Both in 0.1N HCl.

TABLE : 3.1

Samples Analysed for Nucleotides at Various Wavelengths

Wavelength (nm)	IMP found (mg/100 g)		GMP found (mg/100 g)	
	Instant noodle soup	Chicken vegetable soup	Instant noodle soup	Chicken vegetable soup
239	25.10	4.78	21.85	3.26
254	25.15	4.90	22.05	3.28
269	25.07	5.63	21.99	3.26

TABLE : 3.2

Linearity of Peak Area Measurements

Compound	Concentration range tested	Correlation coefficient	Detection limit
Glutamate	0.2-0.025 mg/ml	0.99998	10 µg/ml
Aspartate	0.2-0.050 mg/ml	0.99999	20 µg/ml
PCA	0.2-0.050 mg/ml	0.99999	20 µg/ml
Chloride	4.0-0.025 mg/ml	0.99995	20 µg/ml
IMP	10.0-0.625 µg/ml	0.99999	0.1µg/ml
GMP	10.0-0.625 µg/ml	0.99993	0.1 µg/ml

TABLE : 3.3

Precision of Analysis Procedure for Chicken Vegetable Soup

Compound	Amount found mg/100 g	Average amount mg/100g	Standard deviation
Glutamate	76,80,76,79,76	77.4	1.9
Aspartate	18,18,17,18,18	17.8	0.4
Chloride	1142,1127,1125,1117,1122	1126.6	9.4
IMP	4.98,4.61,4.93,5.11,4.88	4.90	0.18
GMP	3.12,3.36,3.53,3.61,3.57	3.45	0.17

TABLE : 3.4

Precision of Analysis Procedure for Chicken Rice Soup

Compound	Amount found mg/100 g	Average amount mg/100g	Standard deviation
Glutamate	243,239,244,245,245,243	243.2	2.2
Chloride	1714,1738,1700,1726, 1738,1756	1728.7	19.8

TABLE : 3.5

Recovery of Compounds in Consomme Soup

Compound	Amount present	Amount added	Amount found	Recovery %
IMP	592.3 μg	10 μg	602.3 μg	100
		20 μg	613.8 μg	105
		40 μg	633.2 μg	100
		80 μg	673.9 μg	101
GMP	48.8 μg	10 μg	58.6 μg	98
		20 μg	67.3 μg	93
		40 μg	89.6 μg	102
		80 μg	128.9 μg	100
Glutamate	36.0 mg	5 mg	41.0 mg	100
		10 mg	45.8 mg	98
		20 mg	57.3 mg	106
		40 mg	77.0 mg	102
Aspartate	3.0 mg	5 mg	7.9 mg	98
		10 mg	13.1 mg	101
		20 mg	22.8 mg	99
		40 mg	42.7 mg	99
PCA	4.6 mg	5 mg	9.0mg	89
		10 mg	14.1 mg	95
		20 mg	23.7 mg	95
		40 mg	43.3 mg	97

Chloride	130.0 mg	10 mg	139.9 mg	99
		20 mg	150.7 mg	103
		40 mg	168.5 mg	96
		80 mg	211.0 mg	101

TABLE : 3.6

Recovery of Compounds in Rice Soup

Compound	Amount present	Amount added	Amount found	Recovery %
IMP	n.d.	40 μg	40.9 μg	102
		80 μg	81.0 μg	101
		100 μg	96.4 μg	96
		120 μg	115.4 μg	96
GMP	n.d.	40 μg	42.5 μg	106
		80 μg	78.3 μg	98
		100 μg	101.0 μg	101
		120 μg	124.8 μg	104
Glutamate	n.d.	20 mg	19.6 mg	98
		40 mg	39.1 mg	98
		50 mg	50.5 mg	101
		60 mg	59.1 mg	99
Aspartate	n.d.	10 mg	10.1 mg	101
		20 mg	20.7 mg	103
		25 mg	23.7 mg	95
		30 mg	29.0 mg	97
PCA	n.d	20 mg	20.4 mg	102
		40 mg	40.4 mg	101
		50 mg	48.0 mg	96
		60 mg	57.8 mg	96

Chloride	n.d.	40 mg	42.4 mg	106
		80 mg	77.6 mg	97
		100 mg	100.0 mg	100
		120 mg	120.0 mg	100

the buffer as the initial diluting solution. Also, Millipore filtration was used prior to injection of the sample solution into the HPLC system. This helped to remove particulate matter and some color remaining in the sample, extending the HPLC column life.

The HPLC system not only gave an excellent separation of the six compounds studied, but also many other components present in food. This is shown in the Figures 3.5, 3.6, 3.7 and 3.8. This HPLC method represents a rapid, accurate method of analysis of flavor enhancers, salts, aspartic acid, and pyroglutamic acid which should simplify food analysis of these compounds.

3.2 Flavor Enhancers Content of Foods

Many foods naturally contain MSG, IMP and GMP (Tables 1.4, 1.5, 1.11, 1.12). The results given in Table 3.7 represent flavor enhancers found after analysis of a number of foods. Commercial flavor enhancer additives exist in many different forms (see section 1.1.5). The amount of IMP and GMP found in commercial food products (Tables 3.3 and 3.7) reflected the source of flavor enhancers used. Amount of MSG found in the restaurant foods was from 241 to 373 mg/100g which is the normal quantity of MSG added to food (see section 1.1.4). Pyroglutamic acid (PCA) was found in tomato soup, Japanese restaurant miso (soybean paste) soup, consomme soup, tempura sauce (one type of soysauce) and potato chips, but not in other samples. Glutamine was found in a number of processed foods, such as potatoes (164, 187) and in tomatoes (164). Mahdi et. al reported that all glutamine in low acid foods is converted to pyroglutamic acid during thermal processing (119), and the best temperature conditions for conversion of glutamine to PCA is 240°F (35). The chemical reactions of glutamine have been reviewed by Archibald in 1945 (10), and the kinetics of PCA formation was studied by Cleaves (34).

Fig. 3.5 Liquid Chromatogram from UV(I) and RI (II) of Chinese Restaurant Soup at Flow Rate of 1ml/min with 0.017 M, pH 4.0 Phosphate Buffer.

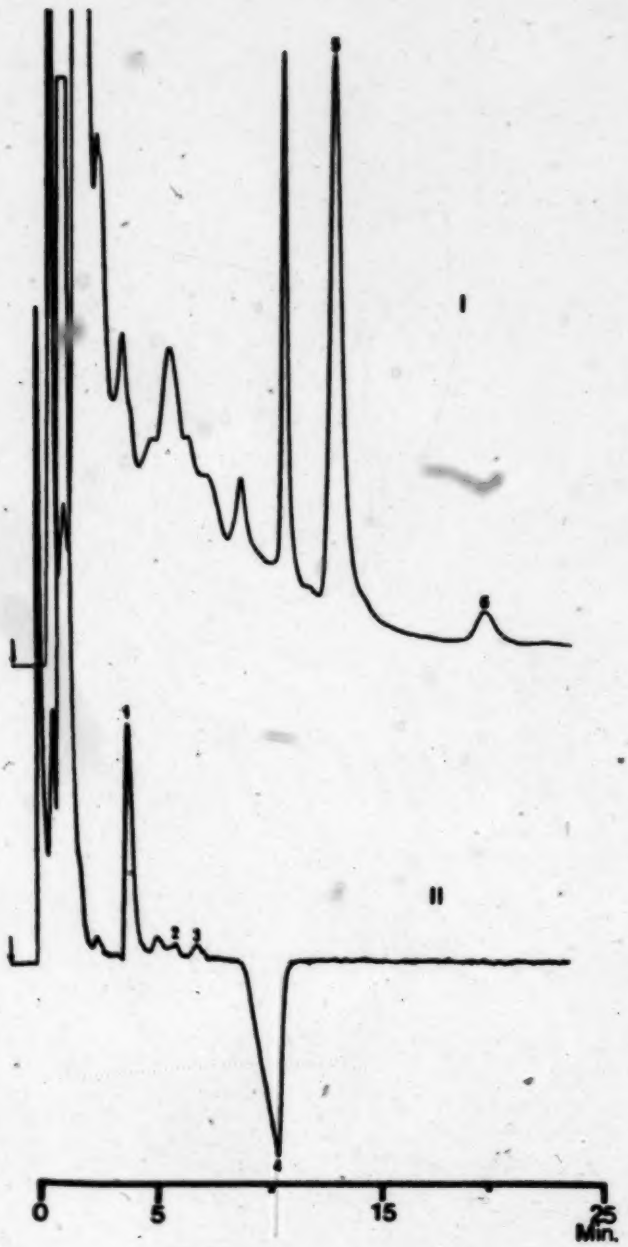


Fig. 3.6 Liquid Chromatogram from UV(I) and RI(II) of Beef Consomme Soup at Flow Rate 1ml/min with 0.017M, pH 4.0 Phosphate Buffer.

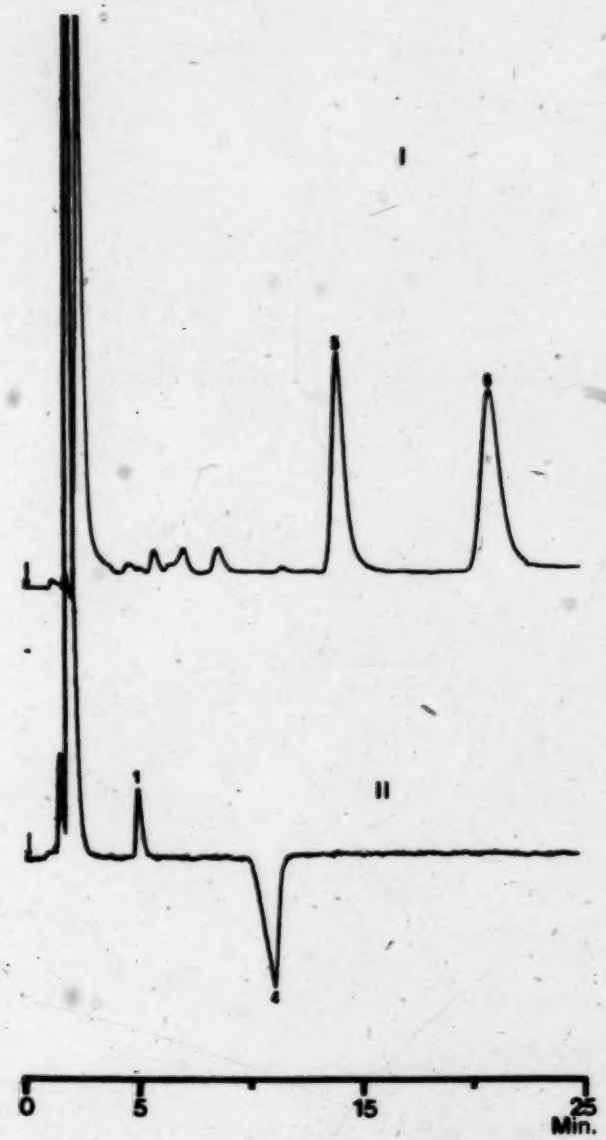


Fig. 3.7 Liquid Chromatogram from UV(I) and RI(II) of Japanese Instant Noodle at Flow Rate of 1ml/min with 0.017M, pH 4.0 Phosphate Buffer.

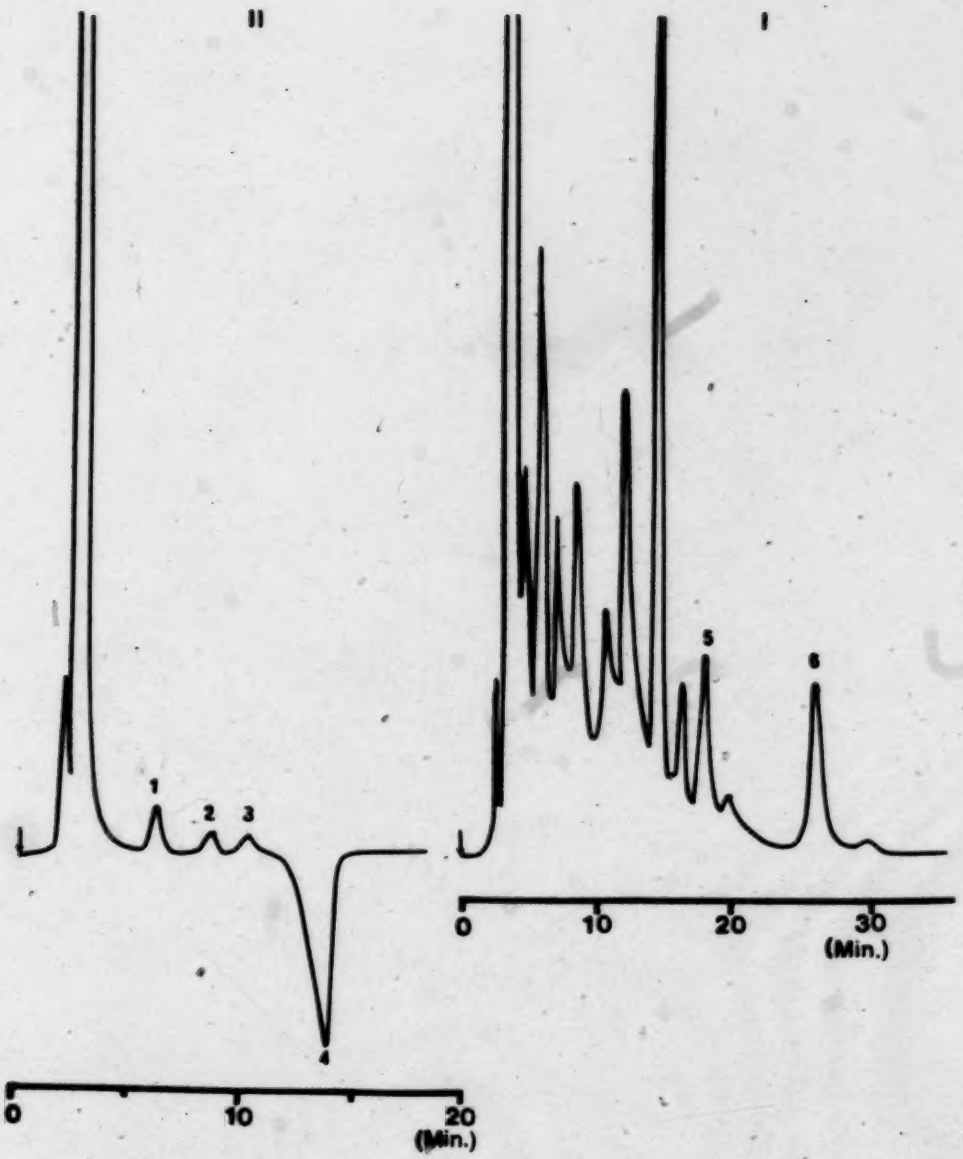


Fig. 3.8 Liquid Chromatogram from UV(I) and RI(II) of Tomato Soup at Flow Rate of 1ml/min with 0.017M, pH 4.0 Phosphate Buffer.

TABLE : 3.7

Foods Containing Flavor Enhancers

Food sample	Glutamate mg/100mg	Aspartate mg/100g	Chloride mg/100g	PCA mg/100g	IMP mg/100g	GMP mg/100g
Tomato soup (canned)	77	41	1015	53	0.94	1.26
Beef consomme soup (canned)	414	30	1300	46	5.95	0.49
Potato chips	191	41	3620	218	n.d	0.94
Japanese instant noodle (dried form)	1035	n.d	5637	n.d	25.15	22.05
Chinese Resturant soup (meat and vegetable soup)	373	n.d	772	n.d	3.47	0.20
Japanese Restaurant soup (miso-seatangle soup)	241	114	1588	59	0.34	n.d
Beef-in-A-Mug	2494	755	33067	n.d	28.12	22.83
Tempura Sauce	1561	460	13675	195	48.80	31.27

Mahdi et. al found a large amount of PCA in processed tomato juice, potatoes, mushroom and beans(119). PCA is quantitatively converted to glutamate in a strong acidic medium at high temperature (199). According to these data, PCA found (Table 3.7), resulted from the original ingredients in the processed foods, and the amount of MSG found was probably independent of the conversion of PCA to glutamate since the acidity of the foods studied was not great enough for the conversion.

3.3 Stability of Flavor Enhancers

Neither glutamic acid nor reducing sugar was found in the examination of casein and starch used for the canning experiment. Also, in the analysis of canned "soup" without flavor enhancers added (group 5), none of the studied compounds was found. Tables 3.8 to 3.11 indicate the recoveries of flavor enhancers in canned samples after analysis (section 2.4). It was obvious that MSG is very stable to canning, and to the interactions with ingredients used, such as sugar, protein and starch. The Maillard reaction was expected to occur between MSG and glucose. There was no significant difference detected in the recovery of MSG between cans containing glucose and those without glucose illustrating that MSG was not lost via the Maillard reaction. Yellowish protein precipitate was found at the bottom of all the cans containing protein (Table 3.12). Although protein probably reacted with sugar in a Maillard type reaction to some extent, the yellow color of protein seemed due to some other chemical reactions, because the can containing protein but no sugar still gave a yellowish protein precipitate (Table 3.12, can No 2-T-1). From data presented in Table 3.12, it seems that the presence of starch reduced the yellowing reaction of protein to some extent, because cans without starch contained much darker yellow-orange protein precipitate. The percent averaged recovery of MSG from various type of "soups" was 96±3. An expected decomposition product of MSG, PCA was not found in any canned samples. Wilson and Cannon showed that the rate of conversion of glutamate to PCA was very slow between pH 4.5 and 8.5, even at elevated temperatures for extended periods (199). From the results of this study, it seems that at

approximately pH 5.5 the conversion rate is indeed slow, even at 124°C (temperature of canning recorded by the thermocouples) for 30 minutes with less than 5% (detection limit for PCA using this analytical method) PCA present.

The behavior of flavor nucleotides compared to MSG was very different. IMP and GMP were labile under the canning conditions (Tables 3.8 to 3.11). The percent recoveries of IMP and GMP calculated from these four tables (Tables 3.8 to 3.11) was found to be 68 ± 5 and 53 ± 4, respectively. However, a 94% recovery of IMP and GMP in canned corn beef was reported (3) using similar canning conditions (120°C for 30 minutes versus 124°C for 30 minutes in this study). Also, Hashida et al reported that the retention of flavor nucleotides was from 64 to 70% in canned green peas, 73 to 74% in canned mushroom (79); and 77 to 88% in canned red crab meat (80) without explaining their canning conditions. They stated that the amount of flavor nucleotides decrease was due to the penetration of these flavor compounds into the solid matrix of canned products. The difference in results between this study and others could be attributed to the difference in the analytical methodology employed, namely HPLC versus an enzymatic method (131).

3.4 Decomposition of Flavor Nucleotides

Nucleosides (inosine, guanosine) are the major compounds obtained from degradation of flavor nucleotides (IMP, GMP). The rate of degradation depends on the sample pH and the heating time (Figure 3.9). The mechanism of phosphate hydrolysis of flavor nucleotides is shown in Figure 3.10. Although chemical phosphate hydrolysis in nucleotide monophosphates has not been reported, many authors have shown that the best conditions for hydrolysis of monophosphate esters occurred around pH 4, where the monoanion has the maximum concentration (15-17, 24-27, 41, 48, 49, 70).

TABLE : 3.8

The Recovery of Flavor Enhancers in Canned "Soup", Group 1

Compound	Amount found	Amount added	Recovery %
MSG	178 mg	189 mg	94
IMP	2726 μg	3780 μg	72
GMP	2178 μg	3780 μg	57

TABLE : 3.9

The Recovery of Flavor Enhancers in Canned "Soup" Without Sugar, Group 2

Compound	Amount found	Amount added	Recovery %
MSG	176 mg	189 mg	93
IMP	2396 μg	3780 μg	64
GMP	1990 μg	3780 μg	53

TABLE : 3.10

The Recovery of Flavor Enhancers in Canned "Soup" Without Protein, Group 3

Compound	Amount found	Amount added	Recovery %
MSG	190 mg	189 mg	100
IMP	2836 μg	3780 μg	75
GMP	2064 μg	3780 μg	55

TABLE : 3.11

The Recovery of Flavor Enhancers in Canned "Soup" Without Starch, Group 4

Compound	Amount found	Amount added	% Recovery
MSG	181 mg	189 mg	96
IMP	2406 μg	3780 μg	64
GMP	1772 μg	3780 μg	47

TABLE : 3.12

Observation of Canned "Soups"

Can numbers	pH (after heating in case of test "T" cans)	Observation of color
1-T-1	5.0	Yellowish protein precipitate
1-C-1	4.8	Same as 1-T-1
2-T-1	4.9	Yellowish protein precipitate
2-C-1	4.7	Same as 2-T-1
3-T-1	5.4	White color, no precipitate
3-C-1	5.1	Same as 3-T-1
4-T-1	5.0	Orange color in both soup solution and protein precipitate.
4-C-1	4.7	Same as 4-T-1
5-T-1	4.8	Yellowish protein precipitate.

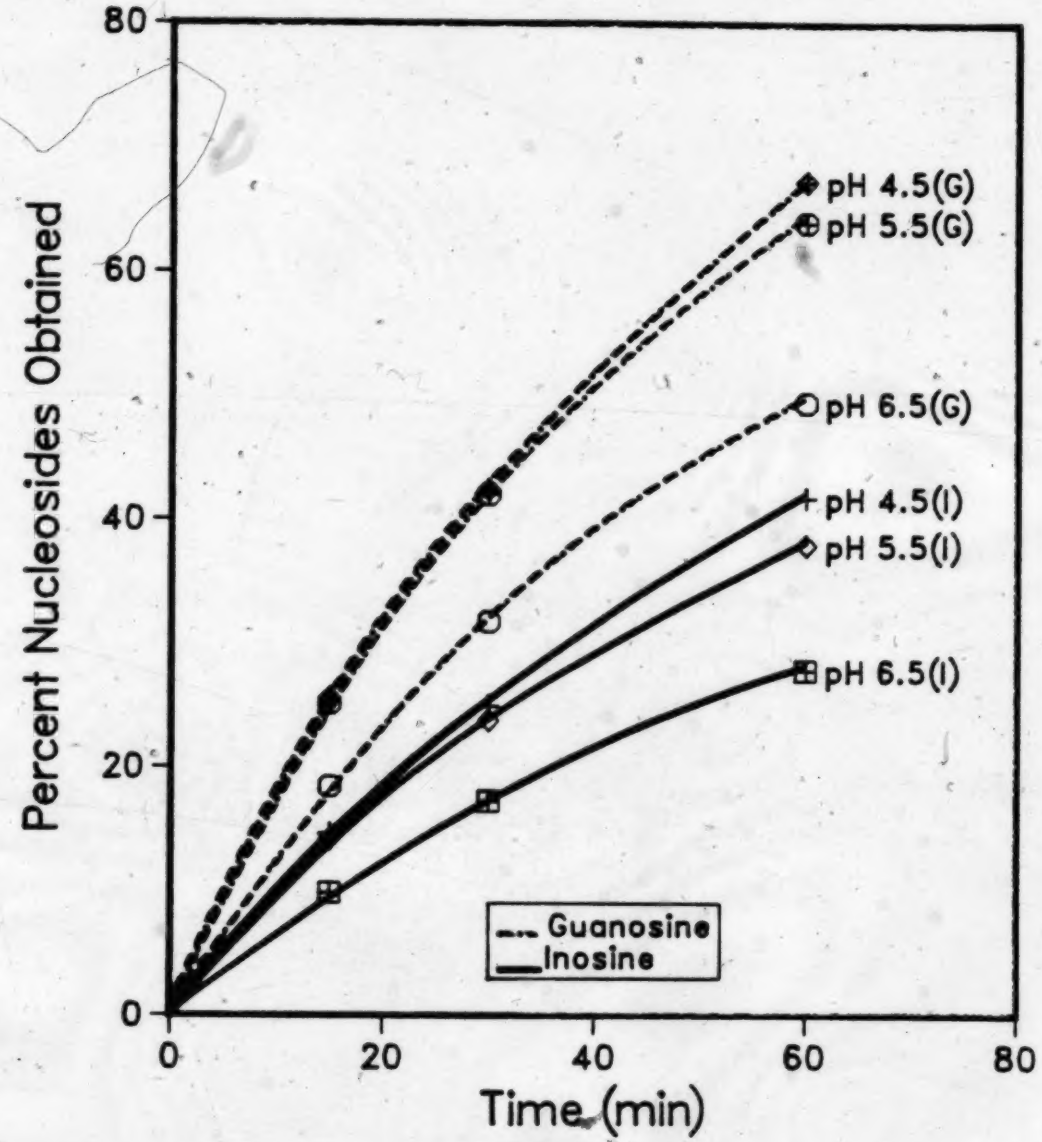
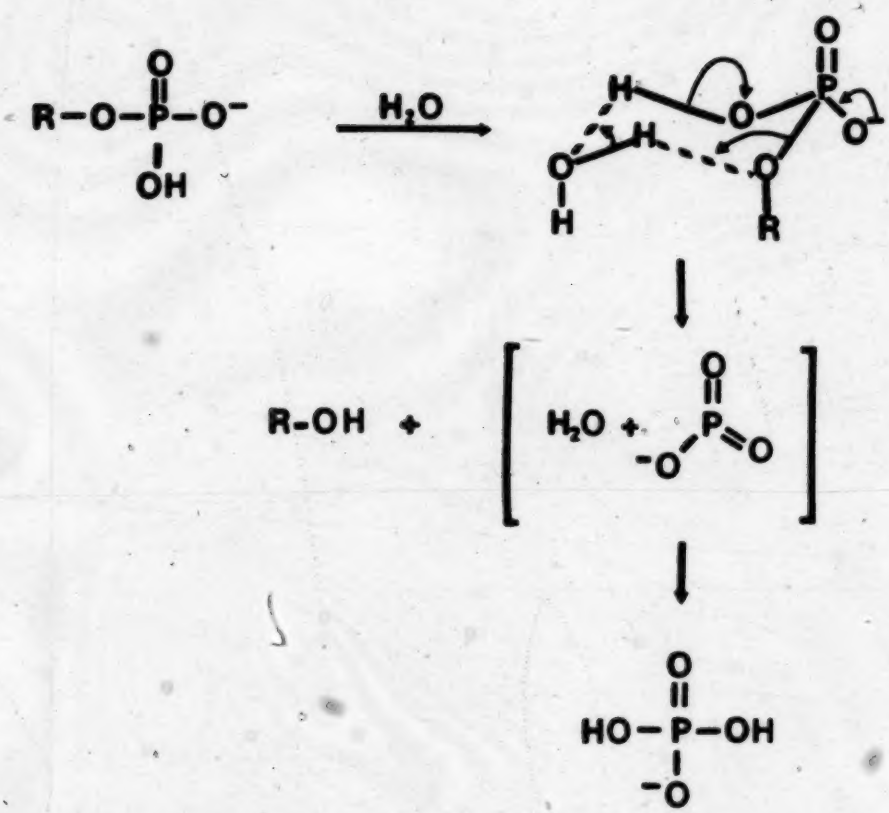
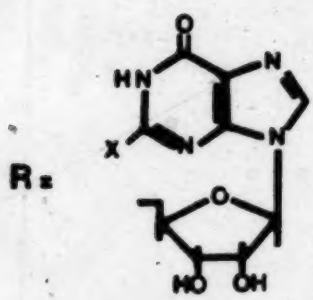


Fig. 3.9 Decomposition of Flavor Nucleotides at Various Retorting Times and pHs





{ Inosine : X = H
{ Guanosine : X = NH_2

Fig. 3.10 Phosphate Hydrolysis of Flavor Nucleotides

The SCX column was found to be very convenient for determination of the nucleosides and the bases which are breakdown products of nucleotides . However, it was difficult to analyse for nucleosides (inosine and guanosine) and bases (hypoxanthine and guanine) simultaneously because complete separation could not be achieved even when a variety of buffer conditions were examined. For example, with a buffer at pH 3.20 the two nucleosides (inosine and guanosine) were separated completely giving sharp peaks but hypoxanthine was eluted right after the guanosine peak and guanine was eluted late giving a very broad peak. The small amount of bases present in the samples as compared to their nucleosides caused difficulty in quantitation. In contrast, it was found that a buffer at pH 3.60 gave the optimum conditions for analysis of the bases, however the two peaks of inosine and guanosine were unresolved.

The remaining undegradated nucleotides (IMP and GMP) in canned samples were determined using a SAX column to obtain accurate values. Although the nucleotides could be separated with the SCX column, they were eluted very near the solvent front with a potential for interference from many compounds. Measurement of peak areas of different concentrations gave a linear response for all compounds analysed (Table 3.13). Upon opening of a canned sample, analysis of both parent compounds (IMP, GMP) and the breakdown products (inosine, hypoxanthine, guanosine, guanine) in the samples were completed within a day thus preventing the possible loss of these compounds (especially the bases) due to microbial growth. Figure 3.11 indicates the complete disappearance of hypoxanthine and guanine in can 60-45-T1, 21 days after the original analysis (also noted in some other cans). Microbial analysis was performed on this sample. The presence of mold, Bacillus cereus , Escherrichia coli , and Staphylococcus aureus were confirmed on selective media and also using a biochemical-test (test performed courtesy Dr. Sunny Lam) according to the methodology described in the Bacteriological Analytical Mannual (60). This microflora obviously (Figure 3.11) caused marked changes in the sample if not analysed rapidly after opening.

TABLE : 3.13

Linearity of Peak Area Measurements

Compound	Column used	Concentration range tested (μM)	Correlation coefficient	Detection limit (μM)
IMP	SAX	6.12-76.5	0.99408	0.25
GMP	SAX	5.89-73.68	0.99716	0.25
Inosine	SCX	7.45-74.56	0.99954	0.50
Guanosine	SCX	7.06-70.60	0.99956	0.50
Hypoxanthine	SCX	1.17-14.69	0.99835	0.05
Guanine	SCX	0.53- 6.62	0.99959	0.25

Fig. 3.11 Liquid Chromatogram of the Canned Nucleotides Solution (60-45-T1) Showing Effect of Microbial Growth on Bases.

A = Original Analysed Sample; B = Sample Analysed 21 Days After. I = IMP G = GMP; I_1 = Inosine; G_1 = Guanosine I_2 = Hypoxanthine; G_2 = Guanine. SCX Column, Phosphate Buffer 0.01M, pH 3.60, Flow rate 0.5ml/min

The level of disodium IMP and disodium GMP used by other workers at pH 5.6 was 0.25 and 0.11%, respectively (3). A level of 0.003% was used in this study to simulate the levels used in the canning industry as shown in Tables 3.3 and 3.7. Samples examined at 0.1% concentration of IMP and GMP (tested using the same canning procedure as other cans with the lower 0.003% levels) gave similar amounts of decomposition of flavor nucleotides. Thus, at least within the concentration 0.1 - 0.003% there was no difference in the decomposition. Buffers of pH 4.5, 5.5 and 6.5 were chosen to include the normal range in pH of food. There was not much change in pH of the sample measured before and after the canning (Table 3.14).

Quantitation of the breakdown products of IMP and GMP was much easier when these two flavor nucleotides were canned separately (Figures 3.12 and 3.13). Precision studies of the parent compounds and their breakdown products were carried out on samples I-45-T1 and G-45-T1 with five repeated injections (Tables 3.15 and 3.16). The total recovery of parent compounds (flavor nucleotides) and their breakdown products (nucleosides and bases) was 91.8 and 92.4% in canned samples of IMP and GMP, respectively. Calculation of these values was based on a comparison with the amount of flavor nucleotides found in the control cans (Tables 3.15 to 3.17).

3.5 Hydrolysis

The possible hydrolysis routes of flavor nucleotides are given in Figure 3.14. The phosphate hydrolysis as stated before (section 3.4), is known to occur almost exclusively from the monoanion as shown in Figure 3.10. Examination of the ionization constants for the flavor nucleotides [Figure 3.15, from TS'O (187)] demonstrates that the maximum concentration of monoanion would occur between the pH extremes of about 6.0 (dianion pKa) and about 1.0 (original pKa of monoanion formation). Probably, like most monophosphate esters (section 3.4), the maximum is near a pH of 4.0. Thus, as canning conditions approach this pH, more and more phosphate hydrolysis would occur.

TABLE : 3.14

Observation of Canned Flavor Nucleotides

Can Numbers	pH		Sample Solution
	before heating	after heating	
15-45-T1	4.80	4.95	Transparent
15-55-T1	5.58	5.52	Transparent
15-65-T1	6.51	6.47	Transparent
30-45-T1	4.83	4.95	Transparent
30-55-T1	5.58	5.55	Transparent
30-65-T1	6.52	6.45	Transparent
60-45-T1	4.75	4.98	Transparent
60-55-T1	5.52	5.55	Transparent
60-65-T1	6.51	6.52	Transparent

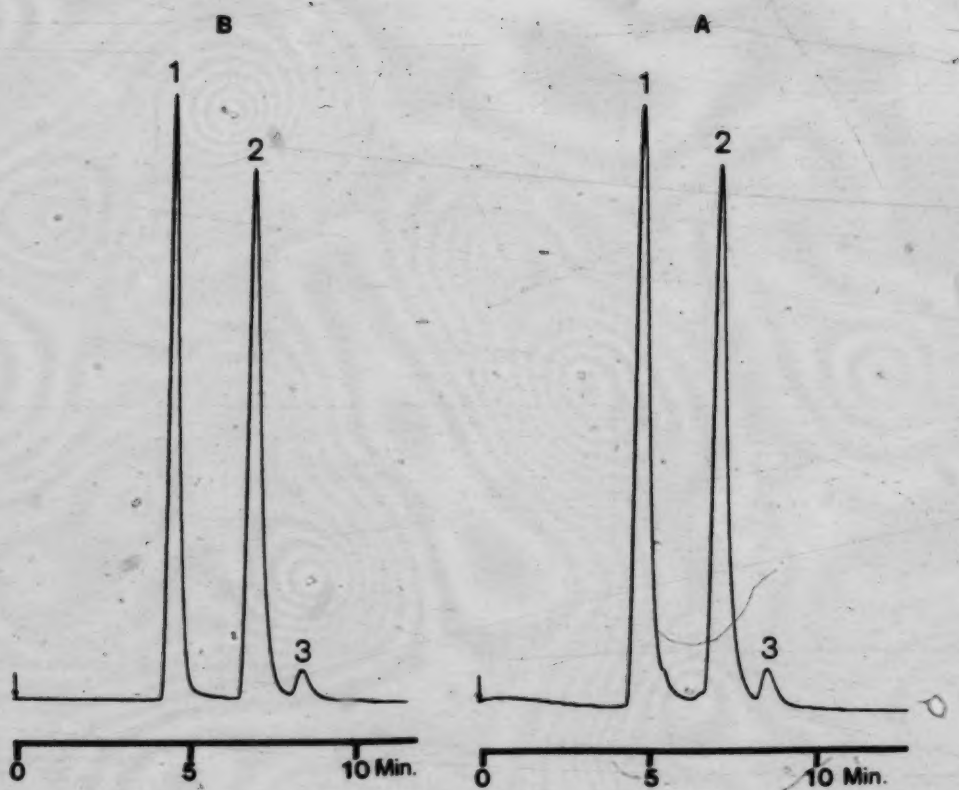


Fig. 3.12 Liquid Chromatograms of A : Canned IMP Solution (I-45-T1) and B : Standards of 1 : IMP (25.5 μM); 2 : Inosine (24.2 μM); 3 : Hypoxanthine (1.5 μM). SCX Column, Phosphate Buffer 0.01M pH 3.60, Flow Rate 0.5 ml/min.

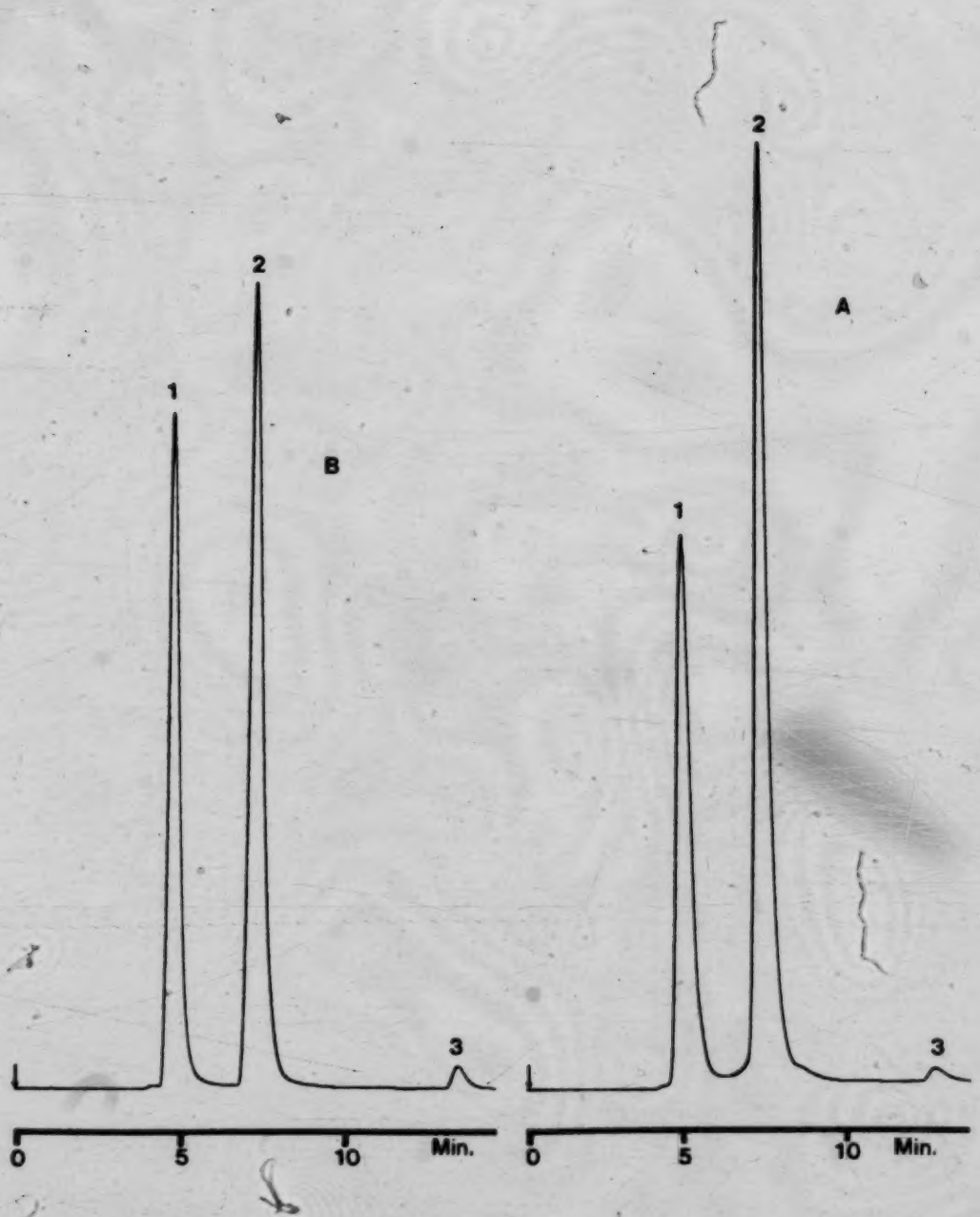


Fig. 3.13 Liquid Chromatograms of A : Canned GMP Solution (G-45-T1) and B : Standards of 1 : GMP (26.5μM); 2 : Guanosine (35.3 μM); 3 : Guanine (1.32 μM). SCX Column, Phosphate Buffer 0.01M, pH 3.60, Flow Rate 0.5 ml/min.

TABLE : 3.15

Precision Analysis of the Degradation of IMP in Canned Samples at pH 4.5

Compound	Column used	Amount found (μM)	Average (μM)	Standard deviation
		Can I-45-T1		
IMP	SAX	37.9,40.0,38.1,40.2,39.2	39.1	1.00
Inosine	SCX	27.8,28.5,27.8,29.0,29.3	28.5	0.68
Hypoxanthine	SCX	1.4,1.7,1.8,1.8	1.7	0.19
		Can I-45-C1		
IMP	SAX	76.9,73.6,75.1,76.0,76.2	75.5	1.30

TABLE : 3.16

Precision Analysis of the Degradation of GMP in Canned Samples at pH 4.5

Compound	Column used	Amount found (μM)	Average amount (μM)	Standard deviation
		Can G-45-T1		
GMP	SAX	27.6,27.9,26.4,27.2,26.0,26.4	26.9	0.76
Guanosine	SCX	40.9,40.5,40.5,40.0,42.1,42.2,40.8	41.0	0.84
Guanine	SCX	0.99,0.95,0.97,0.97	0.97	0.02
		Can G-45-C1		
GMP	SAX	75.9,72.2,76.0,71.5,77.0	74.5	2.5

TABLE : 3.17

Hydrolysis of Flavor Nucelotides in Sample at pH 4.5 Processed at 124°C for 60 min.

Compound	Amount added* (μM)	Amount found (μM)	% Found
	Can I-45-T1		
IMP	75.5	39.1	51.8
Inosine	0	28.5	37.7
Hypoxanthine	0	1.7	2.3
	Can G-45-T1		
GMP	74.5	26.90	36.1
Guanosine	0	41.00	55.0
Guanine	0	0.97	1.3

*Amount found in the control can is considered as amount added.

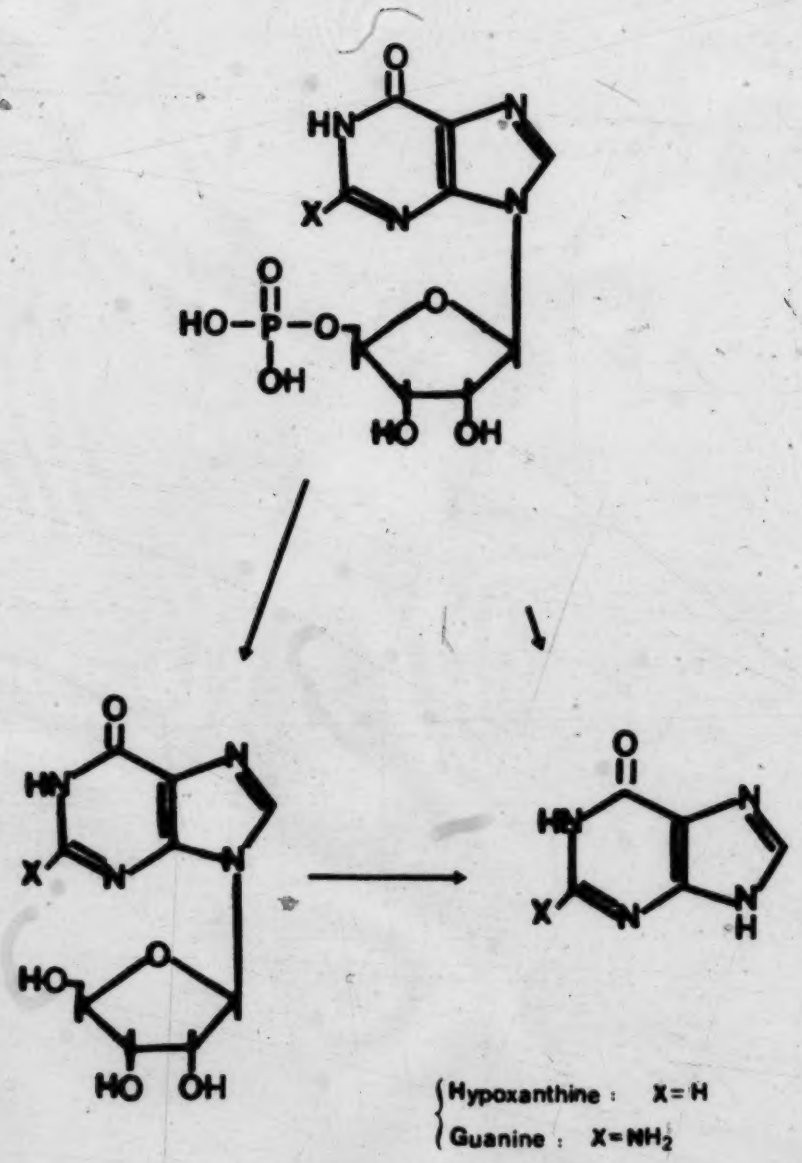


Fig. 3.14 Degradation of Flavor Nucleotides

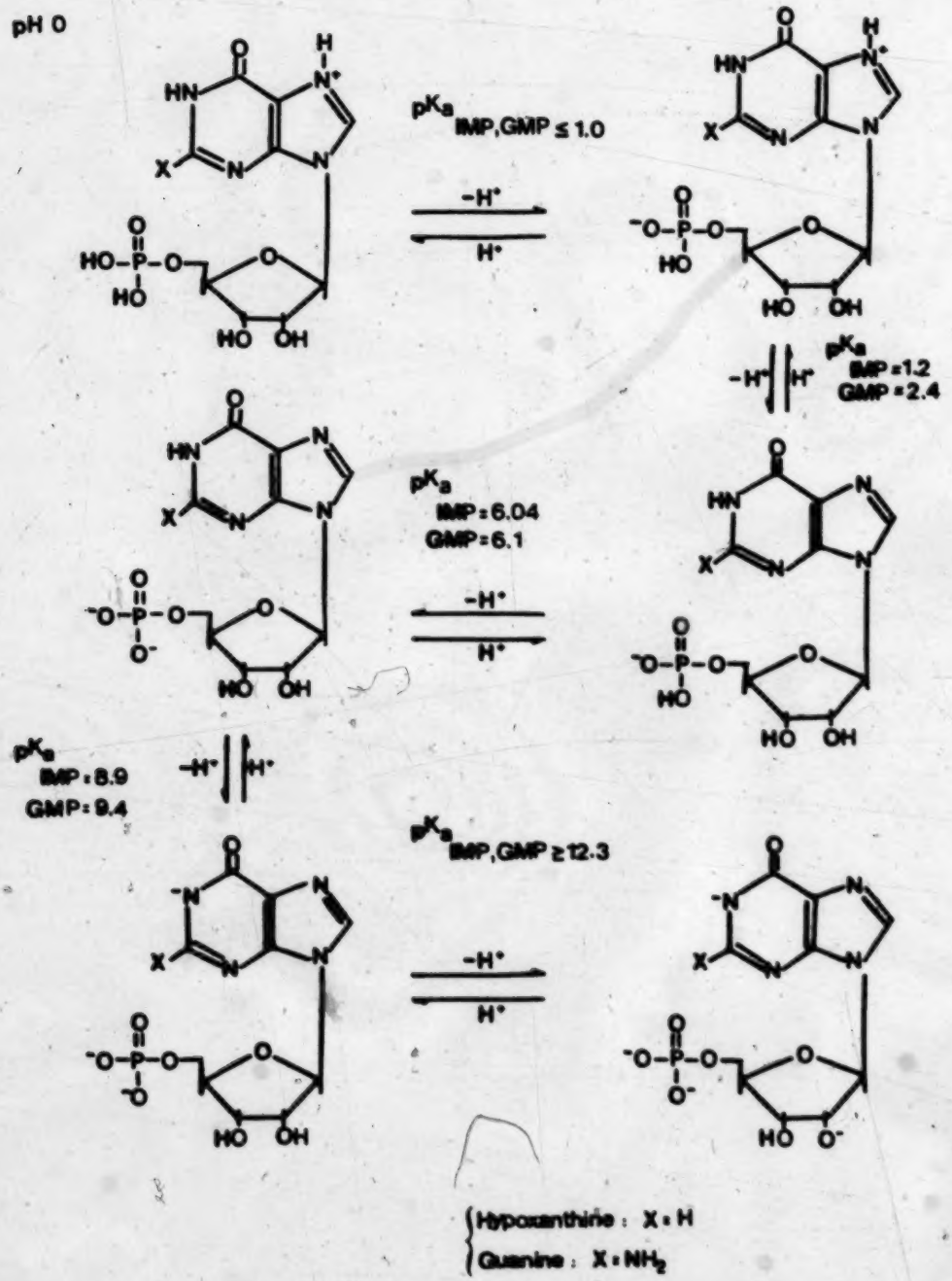


Fig. 3.15 Ionisation Constants of Flavor Nucleotides

To investigate what happens at the pH extremes experimental cannings (temperature 124°C, time = 60 minutes) were carried out at pH 9.0 (0.02M phosphate buffer) and pH 1.5 [established with concentrated phosphoric acid (85%), equal to 0.70M phosphate buffer]. At pH 9.0 no nucleosides or bases were detected, and IMP, GMP were recovered at 93.2 and 93.6%, respectively. At pH 1.5, the canned samples were completely hydrolysed to their bases. Acid hydrolysis of nucleosides and nucleotides has been reported to proceed via two mechanisms (47, 64, 83, 126, 148, 166-168, 174, 212, 213). It is known that in the case of purines (both IMP and GMP are purine nucleotides) the only mechanism operative is the one shown in Figure 3.16. As can be seen by this mechanism, protonation of the bases (pKa IMP = 1.2 and pKa GMP = 2.4) is required as the initial step so a lowering of the pH greatly enhances base hydrolysis. Furthermore, the electron withdrawing effect of the 5'-phosphate would be expected to destabilize the key oxonium ion intermediate leading to slower base hydrolysis for the nucleotides compared with their corresponding nucleosides. This was confirmed by subjecting the nucleosides at pH 4.5 to the canning conditions (Table 3.18). It was found that there was considerably more base hydrolysis from the nucleosides (Table 3.18) than the same concentration of nucleotides (Table 3.17) when both were subjected to similar canning conditions. In fact, with about four times as much base hydrolysis occurring with the nucleosides (comparing Table 3.17 and 3.18, 9.8% hypoxanthine from inosine as compared to 2.3% hypoxanthine starting with IMP, 4.3% guanine as compared to 1.3% guanine starting with GMP), it seems as thought all the base hydrolysis from the nucleotide cans resulted from the nucleosides formed during canning (37.7% inosine and 55.0% guanosine, Table 3.17). Therefore, it seems that the decomposition of the nucleotides during canning occurred by initial phosphate hydrolysis to the nucleosides followed by slow base hydrolysis of the nucleosides as nucleoside levels increased (Figure 3.14).

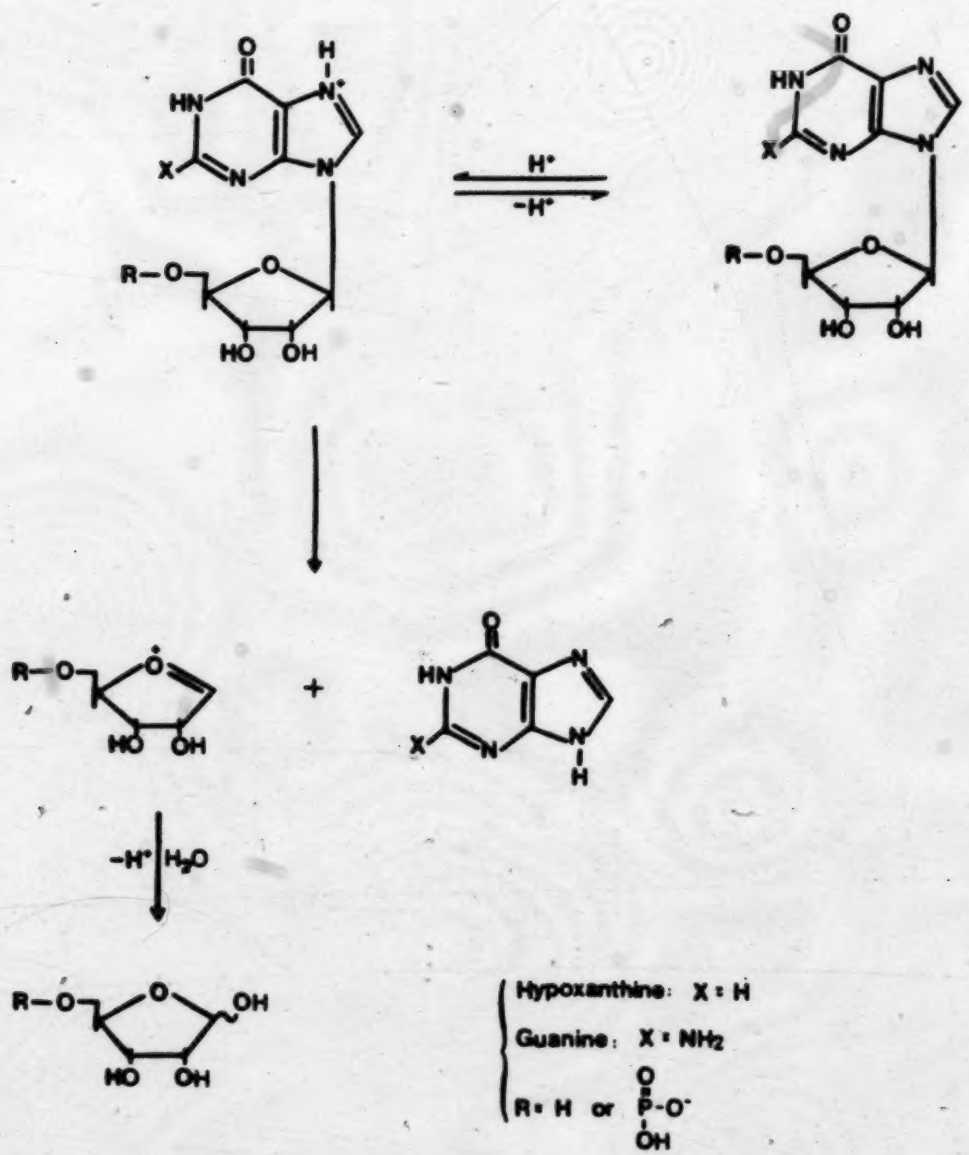


Fig. 3.16 Acid Hydrolysis of Flavor Nucleotides and Their Nucleosides

TABLE : 3.18

Hydrolysis of Nucleosides in Sample at pH 4.5 Processed at 124°C for 60 min.

Compound	Amount added* (μM)	Amount found (μM)	% Found
		Can PSI-45-T1	
Inosine	73.7	66.7	90.5
Hypoxanthine	0	7.2	9.8
		Can-PSG-45-T1	
Guanosine	72.1	68.2	94.6
Guanine	0	3.1	4.3

* Amount found in the control can is considered as amount added.

4. CONCLUSIONS

A modified extraction and HPLC method was developed for simultaneous analysis of flavor enhancers (MSG, IMP and GMP) and other compounds such as aspartic acid, pyroglutamic acid (PCA) and chloride (NaCl) present in foods using an SAX column. This column and/or an SCX column was used for the analysis of 5'-nucleotides and their breakdown products. Various types of commercial canned soups, instant dry mix-soups, consomme soup, soysauce, beef in-a-Mug, chicken in-a-Mug and potato chips, as well as restaurant foods were analysed. The analysis procedures required only 25 minutes with highly reproducible results indicating that HPLC was a very powerful analytical tool for these analyses. These methods could be applied to routine analyses in the food industry and are useful for quality control.

The effect of canning on the stability of flavor enhancers was studied. MSG was very stable with a retention of 94% (after retorting for 30 minutes at temperature of 124°C). Neither PCA, nor evidence of Maillard reaction products, resulting from interaction of MSG with reducing sugar, were found in the samples. Flavor nucleotides (IMP and GMP) are very labile under canning conditions. Their corresponding nucleosides were identified to be the major breakdown products. The amount of inosine and guanosine obtained in canning (retorting 60 minutes at a temperature of 124°C and initial pH of 4.50) were 41.3 and 65.9%, respectively. Nucleosides in the samples were shown to slowly decompose to their bases. The rate of such changes of flavor nucleotides was very dependent on the sample pH. The maximal breakdown of 5'-nucleotides to their nucleosides was observed at pHs approaching 4.0. At a pH of 1.5 flavor nucleotides were hydrolysed completely to their bases. While at a pH of 9.0, they were stable (with a retention of 93%) during the canning process. (retorting 60 minutes, 124°C).

5. RESEARCH NEEDS

Future studies on the changes of MSG in solid foods such as hamburger, bacon, Bar-B-Q products etc requiring high cooking temperatures are recommended.

A detailed study on the influence of pH, water-activity, temperature is required to obtain a thorough understanding of 5'-nucleotide hydrolysis during processing and storage. Prevention of losses to indigenous or added 5'-nucleotides in food under processing conditions is the goal for further studies.

The decrease of 5'-nucleotides, their nucleosides, and the increase of their bases during storage were demonstrated in a study presented in Appendix I and II. Care in future testing is necessary because the presence of the bases may afford good conditions for microbial growth in food.

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APPENDIX I

Effect of Storage on Canned IMP Sample pH 4.5

Days of storage	Sample analysed	IMP added* (μM	IMP found (μM)	Inosine (μM)	Hypoxanthine (μM)
1	I-45-T1	75.5	39.0	28.1	1.54
66	I-45-T2	75.5	29.2	22.5	5.44
92	I-45-T3	75.5	23.2	19.6	6.91

*Amount found in the control can is considered as amount added.

APPENDIX II

Effect of Storage on Canned GMP Sample pH 4.5

Days of storage	Sample analysed	GMP added* (μM)	GMP found (μM)	Guanosine found (μM)	Guanine found (μM)
1	G-45-T1	74.5	26.4	41.3	0.97
66	G-45-T2	74.5	22.4	35.4	1.26
92	G-45-T3	74.5	21.4	37.4	1.51

*Amount found in the control can is considered as amount added.